TOWARDS *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF *CAPSICUM ANNUUM* L. var.'SWEET BANANA'

A thesis submitted for the degree in

Master of Science in Plant Biotechnology

at the University of Canterbury

by

Sakuntala Perera

University of Canterbury

Christchurch

1995

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF PLATES

LIST OF TABLES

ABBREVIATIONS

BA	benzylaminopurine
bp	base pairs
cm	centimetre
DNA	deoxyribonucleic acid
GUS	β-glucuronidase
IAA	indoleacetic acid
IBA	indolebutyric acid
IM	Initiation medium (solid SIM)
kbp	kilobase pairs
L	litre
LB	*Luria* broth
M	molar
mgL^{-1}	milligram per litre
mm	millimetre
MS	Murashige and Skoog medium
NAA	napthaleneacetic acid
nm	nanometre
Ri	root inducing plasmid
RNA	ribonucleic acid
SIM	shoot inducing medium
SIM+K	shoot inducing medium with $50mgL^{-1}$ kanamycin
SIM+K+C	shoot inducing medium with $50mgL^{-1}$ and $200mgL^{-1}$ Claforan
T-DNA	transfer DNA
Ti	tumour inducing plasmid
$\mu Em^{-2}s^{-1}$	micro Einsteins per metre square per second
µl	micro-Litre
v/v	volume per volume
w/v	weight per volume
X-GLUC	5-bromo-4-chloro-3-indolyl-glucuronide

ABSTRACT

The aim of this research project was to develop an *Agrobacterium*-mediated transformation system and a regeneration protocol for *Capsicum annuum* L. ('Sweet banana').

The upper hypocotyl of an 11-day old seedling of this variety formed a rosette of shoot buds when cultured in liquid shoot inductive medium supplemented with 3%(w/v) sucrose and 5mgL^{-1} benzylaminopurine for a period of 20 days.

Shoot formation was inhibited by the antibiotic kanamycin at 50mgL^{-1} or higher. Fresh weight change of the upper hypocotyl decreased drastically at this concentration of kanamycin. A minimum of 4 days culture from the outset in the medium containing 50 mgL^{-1} kanamycin was required for the inhibition of shoot formation. Once shoot induction had occurred transfer to medium having kanamycin did not affect the development of shoot buds.

Explants were inoculated with rapidly growing cultures of three *Agrobacterium tumefaciens* strains and then selection of transformants was carried out by culturing the explants in shoot inductive medium with 50mgL^{-1} kanamycin for 20 days. Attempts to transform the upper hypocotyl explant with *A. tumefaciens* having the binary plasmid pBI 121 (with kanamycin-resistance selectable gene and β-glucuronidase marker gene) were unsuccessful.

Transformation of mature plant tissue with *A. tumefaciens* containing pIG 121 (β-glucuronidase-intron) showed that the variety of *Capsicum* was susceptible to the strains C58::pIG 121 and LBA4404::pIG 121 but not to A4T::pIG 121. Leaf, stem, petal and anther explants expressed GUS activity after 48 hours co-cultivation. Having shown that *C.annuum* L. ('Sweet banana') could express introduced genes, further co-cultivation experiments were carried out with the upper hypocotyl explant. The upper hypocotyl

explant may be recalcitrant as shown by the lack of GUS expression after several trials. Further studies have to be carried out to determine the culture conditions that will render the explant competent.

The shoot bud rosette induced on the upper hypocotyl developed leaf-like structures when cultured on Murashige and Skoog basal medium. Supplementing the basal medium with 0.05mgL^{-1} napthaleneacetic acid and 0.02 or 0.5mgL^{-1} indolebutyric acid produced roots on the excised shoot buds from the rosette but the shoots did not develop further. The optimization for the whole plant regeneration protocol is another area for further research.

CHAPTER 1

INTRODUCTION

1 INTRODUCTION

1.1 Peppers

Peppers are fruits of the plants belonging to the genus *Capsicum* in the family Solanaceae. They are one of the world's major spice crops and are economically significant for individual countries or localised geographic areas where they are grown (Andrews, 1984).

The five domesticated species of the *Capsicum* genus are: the white-flowered *Capsicum annuum* L., *C. baccatum, C. chinense* and *C. frutescens*; and the purple-flowered *C. pubescens* (Morrison *et al.*, 1986).

Capsicum is probably the only cultivated plant with such a great variety of fruit types and has so many different uses over such a wide area of the world. Peppers are an olde-world crop where it was of value in folk medicine (Andrews, 1984). Since the introduction of this crop to the new world its main uses have been as vegetable, spice and condiment. Also capsanthin, a powerful colouring agent present in the fruit, is used extensively in the food industry (Andrews, 1984). Peppers have a distinctly pungent flavour, which is due to an alkaloid compound, capsaicin. This compound is used in pharmaceutical preparations and as a digestive stimulant (Johnson-Sudhaker *et al.*, 1990).

The variety *C. annuum* L. 'Sweet banana' is a mild pepper with a shape, size and colour not unlike its common name implies. As a fresh fruit, it can be used in salads. Peppers in general are a good source of many essential nutrients and especially rich in vitamin A and C (Andrews, 1984).

The typical *Capsicum* plant is a herbaceous annual. *C .annuum* L. ('Sweet banana') begins to flower 12-14 weeks after germination. Plate 1 shows a mature plant and flower. One of the most important aspects concerning crop improvement of *Capsicum* is disease resistance. Many of the domesticated varieties are susceptible to an array of diseases such as bacterial wilt and viruses (Murphy and Kyle, 1994).

1.2 Plant tissue culture requirements

Plant tissue culture involves aseptic *in vitro* manipulation of plant tissue. Parts of a plant such as protoplasts, embryos or micro-cuttings are excised and cultured in a beneficial combination of nutrient media and growth regulators under optimum light and temperature conditions. The pattern of organogenesis can be controlled by adjusting the combination of growth regulators to suit the desired result.

1.2.1 Source and nature of explant

Most plant cells are totipotent; they retain their ability to regenerate new organs even though they have undergone differentiation and acquired specialised functions. This inherent property of plant cells to dedifferentiate and undergo organogenesis makes it possible to carry out tissue culture. The younger the explant, the more readily organ formation occurs *in vitro* (Thorpe and Patel, 1984). Other factors such as genotype and size of explant may also be critical.

1.2.2 Nutritional requirement

The basic nutritional requirements of cultured plant cells are very similar to those normally utilised by whole plants. So the tissue culture media has the same composition of nutrients as the plant requires except allowances for the actual and relative concentration of each mineral are made. For the purpose of plant regeneration and shoot induction, a commonly used medium is Murashige and Skoog or MS medium (Murashige

and Skoog, 1962). The basal medium consists of a balanced mixture of macronutrients and micronutrient elements, vitamins, a carbon source such as glucose or sucrose and organic growth factors with pH maintained between 5.6 and 5.8 (Murashige, 1974).

1.2.3 Growth regulators

Skoog and Miller (1957) discovered that the most critical organic components of plant propagation media are the growth regulators, auxin and cytokinin. These two phytohormones are necessary to induce *de novo* root and shoot formation, respectively. The concentration and ratio of these hormones in the media often controls the pattern of differentiation in culture. A relatively high ratio of cytokinin to auxin favours shoot formation whereas the reverse favours root formation (Skoog and Miller, 1957). This use of hormones has made it possible to propagate a great diversity of plants by tissue culture techniques.

The preferred auxin for tissue culture work is indole-acetic acid (IAA) as it causes less adversity on organ formation, whereas 2,4-dichlorophenoxyacetic acid (2-4-D) is more potent but it strongly antagonises organ development (Murashige, 1974). In the case of cytokinins, 2-isopentyladenine is more effective although 6-benzylaminopurine (BA) and kinetin are nearly of equal effectiveness (Murashige, 1974).

1.3 Importance of plant tissue culture

Research in plant tissue culture not only elucidates on aspects of plant physiology and gene expression, it has applications in the following areas of research: production of secondary metabolites, genetic improvement of crop plants, obtaining disease free clones,

Plate 1. *Capsicum annuum* L. ('Sweet banana'); 4 month old plant and flower

preservation of germplasm and rapid clonal multiplication of selected line through micropropagation (Murashige, 1974).

Capsicum plants produce capsaicin, a secondary metabolite found in the fruit. It has properties that are desirable for use in pharmaceutical and food industries (Andrews, 1984). Work has been carried out on cloned cell cultures to produce this compound (Holden and Yeoman, 1994).

Principally, there are two ways of achieving genetic improvement of a chosen plant species; either through traditional breeding methods or by utilising one of the many gene transfer techniques that have been developed. In peppers, one of the pathological problems that poses a threat to cultivated varieties is viral infection. A means of transferring the viral coat protein to the plant and successfully producing a transgenic plant would have great impact on the industry (Ebida and Hu, 1993).

In domesticated plant species the problem of inbreeding results in a loss of genetic variability. This poses a danger in that a change in cultivation conditions, or a disease outbreak, could result in extensive loss of crops. To ensure that a broad genetic base is maintained for a particular species crossing back to wild parents is vital and this could be carried out by conventional methods or somatic hybridization.

Much work is being carried out to establish propagation protocols in *Capsicum.* This includes work on protoplast and embryo culture that enable somatic hybridisation to be carried out (Christopher *et al.*, 1986; Harini and Sita, 1993).

1.4 Tissue culture in *Capsicum*

Tissue culture of *Capsicum* has been studied by a small number of research groups and there are very few reports concerning the methods of regeneration that are commonly reported for other genera such as *Nicotiana* and *Daucus*. The reason for this could be the lack of success in early attempts to regenerate plants from cultured tissue (Ebida and Hu, 1993). There is even less work published on producing transgenic pepper plants. This in turn could be directly due to the lack of a regeneration protocol. It is important to establish a regeneration protocol and a transformation system that can target the same explant. These two aspects very much dictate the chances of success when attempting to produce transgenic plants.

The successful regeneration of *Capsicum* through tissue culture was first reported by Gunay and Rao (1978). They showed that hypocotyl and cotyledon explants of *Capsicum* can be induced to differentiate into either roots, shoot buds or callus depending on the growth hormone added to the basal medium. Explants of 4 week old seedlings of two varieties of *C. annuum* ('California wonder' and 'Pimento') and a variety of *C. frutescens* ('Bharat') were cultured on MS medium with a combination of the hormones IAA, BA, 2-4-D and napthalene-acetic acid (NAA). Gunay and Rao (1978) observed consistent shoot bud formation on medium containing BA but could not distinguish whether the adventitious buds originated from differentiating explant tissue or from callus. They were able to transfer the plantlets to soil. When the shoots arise from callus, it is not the most desirable way of propagating genetically identical plants because of possible variation occurring in the genome. Plants that are produced from *de novo* axillary bud development show clonal fidelity.

More recently studies have been carried out to clonally propagate *Capsicum* spp. (Christopher and Rajam, 1994). They found that regeneration of shoot-tip explants of

C. praetermissum and *C. annuum* on MS media with BA or Kinetin in the presence of antiauxin TIBA (2,3,5-triodobenzoic acid) resulted in normal diploid plants whereas in the absence of the antiauxin chromosomal aberrations occurred.

Fari and Czako (1981) were able to show that pepper hypocotyl explants produced shoots in media containing BA and IAA. Correlation was established between position of explant on the plant and its morphogenetic response. The morphogenetic response of an explant close to the apical meristem was to produce shoots, the basal explant, close to the root, produced abundant callus and the hypocotyl in the presence of cytokinin produced shoots.

Phillips and Hubstenberger (1985) supported the findings of Gunay and Rao (1978) showing that root and shoot organogenesis in 4 week-old seedlings of *C. annuum* ('California wonder'and 'Yolo wonder') was repeatable and suitable for vegetative propagation. They showed that BA and IAA are the best growth regulators for use with peppers in tissue culture and that shoot formation occurred in the presence of cytokinin with or without IAA or IBA. Non-meristematic explants such as the hypocotyl responded for one month under tissue culture conditions of 25°C and 16 hours photoperiod without any difference between using sucrose or glucose as the carbon source.

Sripichitt *et al.* (1987) found that the age of the *Capsicum* explant used was an important factor affecting the number of shoots formed per explant. Using *C. annuum* L. ('Yatsufusa'), a red pepper, they observed that the frequency of shoots and the number of shoots per explant decreased with the age of seedling and that the 12 day-old seedling yielded the best result. Sripichitt *et al.* (1987) also showed that a BA concentration between 3-7mgL^{-1} yielded the largest number of shoots. This was the first paper to establish the frequency of shoot formation. Shoots are thought to arise from the single

epidermal cells as first observed by Broertjes and Van Harten (1978; in Sripichitt *et al.*, 1987). Further work by Agrawal *et al.* (1988) on *C. annuum* ('Mathania' and 'Bharat') shows that in the presence of cytokinin (BA or Kinetin) shoots are formed *de novo* directly from explant tissue and not from a callus phase.

Agrawal *et al.* (1989) reported optimum shoot induction at $5mgL^{-1}$ BA, but shoot elongation did not occur. Shoots were then placed on medium containing IBA or NAA to obtain whole plants.

Arroya and Revilla (1991) found that the hypocotyl explant of two bell pepper varieties showed highest percentages of organogenesis and number of shoot buds per explant when cultured for 15-20 days on MS with BA or Zeatin. Shoot buds formed in rosettes and these could be rooted on $0.1mgL^{-1}$ NAA and $0.05mgL^{-1}$ IBA.

Whole plant regeneration of *C.annuum* ('Early California Wonder') has been achieved by Ebida and Hu (1993). The plants were developed from shoots formed on hypocotyl explants of 13-day old seedlings. The shoots were excised and rooted on MS medium with 0.5 mgL^{-1} IAA or 0.4 mgL^{-1} NAA. They observed that the shoot buds regenerated directly from the explant without an intervening callus phase and so eliminating the phenomenon of somaclonal variation. Ebida and Hu (1993) suggest that this is an important feature for transformation work.

Ramage (1994) developed a shoot induction protocol for *C.annuum* L. ('Sweet banana'). This study found the upper hypocotyl explant to be the most suitable for shoot initiation. The upper hypocotyl segment of an 11 day old seedling formed distinct shoot primordia at the cut ends when cultured in the shoot inductive medium whereas the lower hypocotyl rarely developed shoots. Ramage (1994) confirmed earlier findings (Agrawal *et*

al., 1989) that the optimum concentration of BA for shoot induction is $5mgL^{-1}$. MS medium having $5mgL^{-1}$ BA and 3%(w/v) allowed 80-100% of the explant to form shoot buds. A minimum of 8 days in shoot induction medium was required for shoot bud development. Although callus was formed as part of the wound response the rosette of shoot buds that developed at the cut ends and arose directly from the superficial layer of the explant (Ramage, 1994).

Other studies to regenerate plants from *Capsicum* spp. includes work by Saxena *et al.* (1981), Diaz *et al.* (1988) and Murphy and Kyle (1994) using protoplast isolation and culture. The initial work by Saxena *et al.* (1981) resulted in regenerated flowering plants and Murphy and Kyle (1994) successfully isolated and inoculated the protoplasts of five genotypes using viral RNA from viruses that are thought to be the most destructive *Capsicum* viruses. Diaz *et al.* (1988) established efficient and reproducible methods for somatic hybridization of protoplasts for crop improvement; however the varieties used were different to 'Sweet banana'.

The attraction for hybrid pepper varieties has led to several reports on the induction of haploid plants. Dumas de Vaulx (1977; in Reynolds, 1986) observed that haploid plants were evident in some experimental lines. They determined by histological techniques that the haploid embryos originated from the synergid containing haploid nuclei and did not degenerate. They obtained haploid plants from anther culture at a low frequency. About 1-3 embryoids per 100 could be produced if anthers in the first stage of mitosis were pretreated at 4°C for 48 hours. Embryo culture has been used in red pepper to increase the multiplication rate in tissue culture (Christopher *et al.*, 1986).

George and Narayanaswamy (1973) reported haploid plants through anther culture in *C.annuum* var.'Grossum'. Agrawal and Chandra (1983) reported formation of multiple

shoots per embryo originating from the margins of the expanded cotyledon and not from the intervening callus phase. When subcultured with $5mgL^{-1}$ BA shoot proliferation and maturation into complete plants occurred. Direct regeneration is important for clonal multiplication as it preserves the ploidy level. A similar result was reported by Harini and Sita (1993) who were able to develop plants from immature zygotic embryos through direct somatic embryogenesis without the intervening callus phase.

A rather different way of regenerating a plant was reported by Valero-Montero and Ochoa-Alejo (1992). The group rooted hypocotyls first and then inverted it into MS with $5mgL^{-1}$ BA and $0.3mgL^{-1}$ IAA to yield maximal bud induction rates of 46-100%.

Effect of growth regulators and tissue culture techniques on the pattern of organogenesis in *Capsicum* spp. differs vastly between the different varieties (Sripichitt *et al.*, 1987; Gunay and Rao, 1978), showing that genotype is a critical factor in influencing organogenic response. As the varieties differ in their response to *in vitro* manipulations a regeneration protocol designed for one variety may not be applicable to another variety (Liu *et al.*, 1990).

1.5 Genetic transformation in higher plants

Newly developed transformation methods in plants will not only provide new insight into important physiological and developmental processes but also have the potential for improving the agronomical performance of crop plants (Fraley *et al.*, 1986).

In general, the development of successful systems for producing transgenic plants depends on two aspects: (i) the development of a system to deliver genetic material, usually DNA, into a plant cell and (ii) the development of cell, organ or tissue culture

technique that permits selection of the transformed plant material and the regeneration into a whole plant.

Transformation of plant cells has been achieved by many different techniques, from the technically sophisticated biolistics approach to membrane disruption by chemicals enabling DNA uptake (Fehér *et al.* 1991). Soil bacteria that carry out DNA transfer into plant cells can be exploited in the laboratory. This is technique will be the focus of this research.

1.6 *Agrobacterium*- mediated gene transfer

Bacteria of the genus *Agrobacterium* are free living, opportunistic soil bacteria that have evolved the unique capacity to interact genetically with susceptible plants. The interaction results in the stable insertion of a defined part of the bacterial genome into the plant genome. It is this ability of the bacterium to genetically transform the plant tissue that is the basis of all gene transfer technology that exploiting this system (Binns and Thomashow, 1988; Hooykaas, 1989; Potrykus, 1990; Zambryski, 1992). Infection by *Agrobacterium tumefaciens* causes tumorous plant growth commonly called crown galls and *A. rhizogenes* infection leads to hairy root disease.

1.6.1 *Agrobacterium tumefaciens*

The ability of *A. tumefaciens* to form tumours depends on the tumour inducing (Ti) plasmid. The Ti plasmid is approximately 200kbp in size and contains a small region between 15 and 30kbp, referred to as transfer DNA or T-DNA. This T-DNA is transferred to the plant cell and covalently integrated into a plant chromosome (Chilton *et al.*, 1980; Willmitzer *et al.*, 1980). The Ti plasmid also has a region outside the T-DNA referred to as the virulence region carrying genes (*vir* genes) that are involved in tumour induction (Winans *et al.* 1987). The expression of the *vir* genes may be required for the

conditioning of the plant cells during infection and for the subsequent transfer of the T-DNA (Yanofsky *et al.* 1986). Wild type T-DNA in native Ti plasmids has a region that encodes for the synthesis of the plant growth hormones; auxin and cytokinin. Over-production of these hormones manifests as a tumorous phenotype. This region also encodes for compounds called opines that are metabolic substances for the bacteria. The nature of T-DNA transfer is not fully elucidated but molecular characterisation has shown that it is defined and delimited by two 25bp direct repeats (Wang *et al.* 1984; 1987). It is the DNA present between these two borders that is transferred (Caplan *et al.*, 1983; Zambryski *et al.*, 1983). As the right border is critical (Wang *et al.*, 1984) it would suggest that T-DNA transfer starts from the right and so the border sequence directs polar transfer.

The T-DNA itself does not encode *trans*-acting functions required for its transfer (Lichtenstein, 1986). By cloning foreign DNA into the T-DNA region it is possible to exploit the natural ability of *Agrobacterium* to transfer the DNA into the plant genome.

1.6.2 *Agrobacterium rhizogenes*

A. rhizogenes generally induces adventitious root formation in the wounded tissue. This ability is conferred by the root inducing (Ri) plasmid. The T-DNA region of the Ri plasmid can also be used for transformation work (Bercetche *et al.*, 1987; Tepfer, 1984; 1990). The plasmid and the transfer region are similarly denoted as with *A. tumefaciens* and although homologous (Huffman, *et al.*, 1984), differences exist at the molecular level (De Paolis *et al.*, 1985).

1.6.3 Host Range

A wide range of plants are susceptible to tumour and hairy root formation induced by *Agrobacterium.* These include mainly dicotyledonous plants, a few gymnosperms (De Cleene and De Ley, 1976) and some monocotyledonous plants (Dommisse *et al.*, 1990).

The host plant plays a key role in the infection process. Exudates produced by wounded plant cells are able to induce the expression of the bacterial encoded virulence genes. This induction is critical to the DNA transfer process. The signal molecules present in a commonly studied and routinely transformed plant *Nicotiana tabacum*, have been purified and identified as the phenolic compounds acetosyringone (AS) and α - hydroxy- acetosyringone (OH-AS). Different plant cell types produce these molecules varying levels. Wounded plant cells produce AS and OH-AS in greater amounts thus *Agrobacterium* has evolved to respond to these compounds specifically representative of plants susceptible to transformation (Stachel *et* al., 1986a & 1986b).

Agrobacterium has been used to transform major crops such as soybean, cotton, sugarbeet, sunflower and oilseed rape within the first decade of developing the technique. Since then transformation of a variety of crops including *Cucumis melo* L. (Fang and Grummet, 1990), *C. sativus* L. (Chee, 1990), *Solanum melongena* (Rotino and Gleddie, 1990), *Daucus carota* (Balestrazzi *et al.*, 1991), *Eagopyrum esculentum* Moench. (Miljus-Djukie, 1992) and *S. tuberosum* L. (Conner *et al.*, 1991 and Filho *et al.*, 1994), has been achieved.

1.7 Development of vectors with selectable markers

Once the components of the Ti plasmid were mapped, genetic manipulations were made possible enabling the construction of a transformation vector. The Ti plasmid was

disarmed by substituting foreign genes for the tumour inducing genes (Fraley *et al.*, 1983 and Zambryski *et al.*, 1983) and the first practical system for genetic engineering was thus assembled. In the absence of the tumour inducing genes plant cells could be regenerated into normal and fertile plants. However without the tumour phenotype, genetically transformed cells would have to be identified by other means (Bevan, 1984).

A critical step in the development and evaluation of transformation strategy for plants is the use of vectors with genes that can act as dominant selectable markers (Fraley *et al.*, 1983 and Herrera-Estrella *et al.*, 1983). Under various selection pressures, these genes provide a growth advantage to cells that integrate the vectors and express the marker gene. Because transformation events may occur at a low frequency an efficient, clear cut selection system is needed to detect transformants, in order to be able to recover or separate them from the untransformed explants (Fraley *et al.*, 1986). Therefore vectors used in transformation protocols must have two integral components: regulatory sequences to enable gene expression in plant cells and marker genes for selection.

To achieve constitutive expression in various tissues of the plants, the coding regions of the marker genes have been fused to promoters or other regulatory sequences known to function in plants such as those from nopaline synthase (NOS) or octopine synthase (OCS) genes of the Ti plasmid and from the Cauliflower Mosaic Virus (CaMV) 35S or 19S transcript. The 35S CaMV acts constitutively ensuring that high level of transcription occurs at all times in most cell types. But it has been reported by Jefferson *et al.* (1987) that this promoter may be dependent on cell types.

Fraley *et al.* (1983) constructed a chimeric marker gene with the required components. It consisted of the regulatory sequences of the *Agrobacterium* encoded nopaline synthase gene and the neomycin phosphotransferase gene (NPT-II) from the

bacterial transposon Tn5. The NPT-II codes for an enzyme that catalyses the transfer of a phosphate moiety from adenosine triphosphate (ATP) to a number of aminoglycoside antibiotics including kanamycin, thereby detoxifying them. The antibiotic kanamycin inhibits protein synthesis in prokaryotic cells and affects plant cells because it recognises the protein translation mechanism in the 'prokaryotic-like' organelles chloroplast and mitochondria present in the plant cells. (Wilmink and Dons, 1993). The most visible effect on plants is chlorosis; a bleaching of the green tissue caused by lack of chlorophyll synthesis (Pollock *et al.*, 1983).

The concentration at which an antibiotic will repress cellular activity without killing the tissue must be determined and then used as the selection force. At that critical concentration of an antibiotic such as kanamycin, transformed cells can grow and undergo organogenesis; whilst most non-transformed explants could not (Colby and Meredith, 1990).

A reporter gene is also present on a vector and this codes for an enzyme that allows sensitive and rapid detection of transformed cells rather than providing selection under pressure. These genes are important for monitoring transformation and detection of promoter activity. One such reporter gene is the β-glucuronidase gene (uidA locus) of *Escherichia coli* (Jefferson, 1987) that allows great sensitivity of detection. The enzyme is encoded by this gene is a hydrolase that cleaves a variety of β-glucuronides. Enzyme activity can be detected histochemically in cells using substrates such as 5-bromo-4-chloro-3-indolyl glucuronide (X-GLUC) and results in blue colouration of cells where the enzyme is expressed. Such an assay, commonly referred to as GUS assay, offers great precision in identifying the specific cells and tissues in which the promoter is active in transgenic plants or to establish which cells or tissue are competent for T-DNA uptake (Jefferson, 1987).

Autonomously replicating vectors have been constructed (Hoekema *et* al., 1983; Bevan, 1984; An *et al.*, 1985 and Ozcan *et al.*, 1992) so that they can be maintained in *E. coli* for ease of manipulation or in *Agrobacterium*. These vectors referred to as binary vectors do not have to integrate with the resident Ti plasmid of *A. tumefaciens* and the *vir* functions are provided *in trans* by the Ti plasmid.

1.8 Development of binary vectors pBI 121 and pIG 121

The binary vectors pBI 121 (Jefferson *et al.*, 1987) and pGI 121 (Ohta *et al.*, 1990) were used in this research project as the vectors carrying the dominant selectable marker genes for kanamycin resistance and the β-glucuronidase gene. Both were derived from the 10kbp plasmid Bin 19 (Bevan, 1984).

Figure 1 illustrates the modifications made to pBin 19 to obtain pBI 121 and pIG 121. Jefferson *et al.* (1987) ligated the coding region of β-glucuronidase gene, 5' of the nopaline synthase polyadenylation site of pBin 19. The CaMV 35S promoter was added resulting in a chimeric gene to create pBI 121.

Indicator genes maybe expressed in *Agrobacterium* and this may interfere with the precise determination of timing and localisation of T-DNA transfer (Vancanneyt *et al.*, 1990). To prevent this expression in *Agrobacterium* Ohta *et al.* (1990) and Vancanneyt *et al.* (1990) have modified the GUS gene by introducing a plant intron. Only correct splicing of the intron would give rise to GUS enzyme activity. Due to the lack of eukaryotic splicing apparatus in *Agrobacterium*, expression would not occur in the bacteria and any expression detected would be from plant cells that had incorporated the T-DNA. Ohta *et al.* (1990) modified the GUS gene of pBI 121 by inserting a 190bp intron giving rise to pIG 121. A stop codon in the intron in the same reading frame as the

enzyme prevents the expression of GUS unless it is spliced out. They observed that detection of GUS activity in genetically modified plant cells was possible within 2 days after inoculation with *Agrobacterium* having pGI 121. The use of this plasmid decreases the time required for assay hence most explants could be tested for competency of T-DNA uptake without having to first establish a selection or regeneration protocol.

1.9 Co-cultivation

To enable *Agrobacterium* interaction with explants the explants are incubated with a bacterial inoculum. After this co-cultivation plant cells are washed free of the contaminating bacteria and then cultured in selection and regeneration media. The selection media also contains an antibiotic such as Claforan to suppress any bacteria that may be present.

During co-cultivation the *vir* genes are induced in *Agrobacterium* and the bacteria bind to the plant cells around the wounded edge of the explant and the T-DNA transfer can occur. The time required for the process may be 24 to 48 hours (Lichtenstein and Fuller, 1987). Horsch *et al.* (1985) were the first to develop a method to routinely transform and regenerate leaf discs of petunia, tobacco and tomato by co-cultivation with *Agrobacterium*.

1.10 *Agrobacterium*-mediated transformation in *Capsicum*

There are few reports on the interaction of *Agrobacterium* with pepper tissues. The susceptibility of *C. annuum* L. to *Agrobacterium* strains B6 and chrIIB was first indicated by De Cleene and De Ley (1976). To date the only work published on *Agrobacterium* induced gall formation and regeneration of transgenic tissue has been Liu *et al.* (1990). They obtained shoot-like structures that expressed introduced genes in

C.annuum L. ('Yolo Wonder L'), a bell pepper. However they were unable to regenerate these shoot-like structures into elongated shoots to yield transgenic plants.

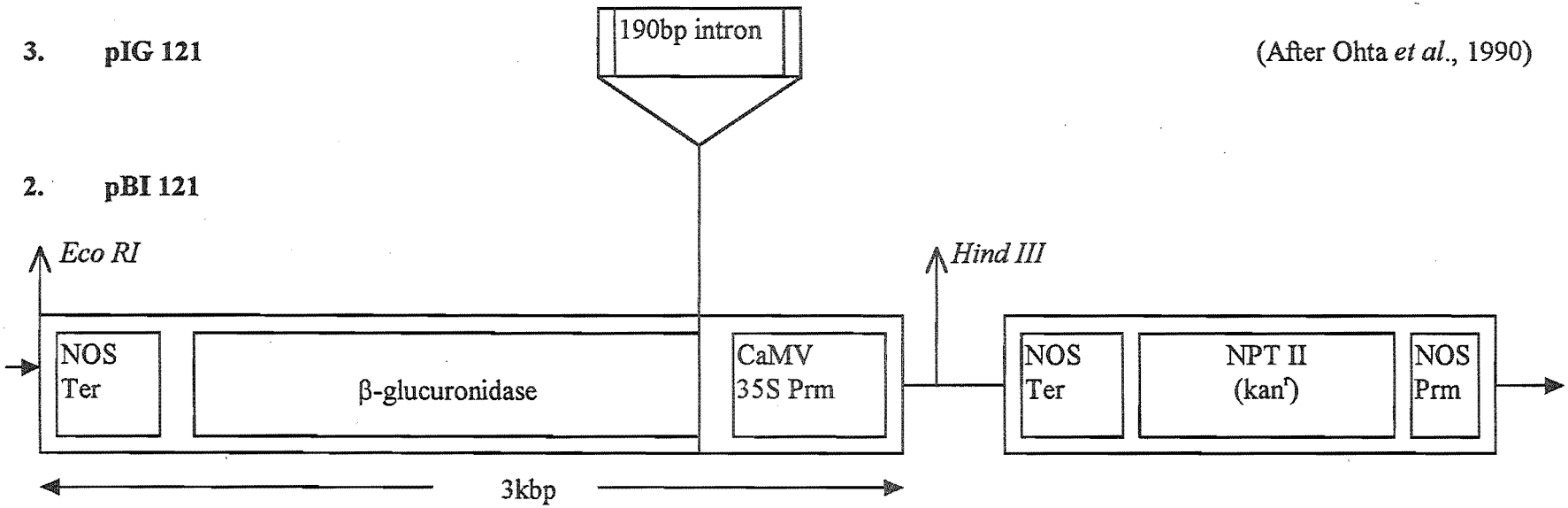
Liu *et al.* (1990) tested 6 cultivars to study effects of genotype on callus formation and regeneration. They selected 'Yolo Wonder L' after preliminary experiments because the proportion of explants forming leaf-like structures was significantly greater for this cultivar on modified MS medium supplemented with $1mgL^{-1}$ IAA and either $2mgL^{-1}$ or $10mgL^{-1}$ BA. In further tests they showed that *C.annuum* was most susceptible to *A. tumefaciens* C58 strain. The vector used was a plasmid derived from pBI 121 (Jefferson *et* al., 1987). An overnight culture of *Agrobacterium* was used as the inoculum, and the explants from 12-14 day old seedling and leaves from 25 day plant was incubated for 24-48 hours at 28°C in the dark. After co-cultivation, explants were placed on selection and regeneration medium. GUS assays were carried out on kanamycin resistant tissue following several subcultures to remove contaminating bacteria.

Liu *et al.* (1990) found that production of kanamycin resistant cell lines was more effective for cotyledon and leaf explant than for hypocotyl segments. They observed that leaf-like structures and shoot buds formed in greater numbers when selection pressure was decreased from 200 μgml^{-1} to 150μgml^{-1} kanamycin. However many of these proved to be untransformed escapes shown by the lack of GUS activity. GUS activity in transformed tissue was localised in vascular and perivascular tissues supporting the findings of Jefferson *et al.* (1987) that under the control of the CaMV 35S promoter the GUS gene shows preferential expression in these particular tissue types.

Figure 1 Derivation of pBI 121 and pIG 121 (not drawn to scale). Both these binary vectors originate from pBin 19 (Bevan, 1984). pBin 19(**1**) was modified by the insertion of a 3kbp GUS expression cassette within the Lac-polylinker site (Jefferson *et al* .,1987) resulting in the vector pBI 121. The GUS cassette (**2**) consists of Cauliflower Mosaic Virus 35S promoter (CaMV 35S Prm), β-Glucuronidase gene and Nopaline Synthase terminator (NOS Ter). Ohta *et* al.(1990) modified it further by inserting an intron into the GUS gene (**3**). Abbreviations: Kan^r, kanamycin resistance; bp, basepairs; kbp, kilobasepairs; LB, left border and RB, right border.

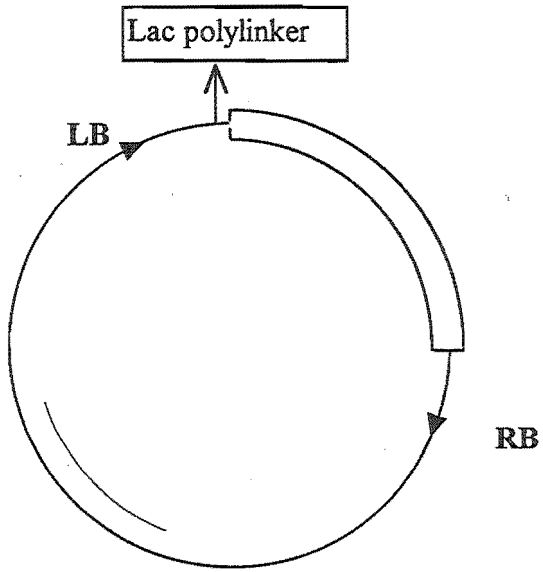
3. pIG 121 (After Ohta *et al.*, 1990)

2. pBI 121



(Jefferson *et al.*, 1987)

1.



pBin 19 (10kbp) (Bevan, 1984)

1.11 Aims and objectives

The aim of the project was to develop an *Agrobacterium*-mediated transformation system for *Capsicum annuum* L. 'Sweet banana' and to define a regeneration procedure to establish transgenic plants.

The target tissue for transformation was the upper hypocotyl of an 11-day old seedling. This explant forms shoot buds *de novo* in shoot inductive medium. Before carrying out the transformation work, the sensitivity of the explant to kanamycin had to be determined. A regeneration protocol had also to be developed to produce transgenic plants from the transformed shoot buds. Once the transformation and regeneration procedures are established a number of factors influencing transformation can be evaluated to optimise the procedure.

The main objectives in this research included:

1. Development of selection protocol to identify transgenic tissue from untransformed tissue;
2. Development of regeneration protocol to establish transgenic plants from the transformed shoot buds;
3. Transformation of explants and optimising the procedure by varying inoculation and co-cultivation periods, inoculum density and pre-conditioning of explants before inoculation;
4. Compare transformation competency of explants from seedling and mature tissue.

CHAPTER 2

MATERIALS AND METHODS

2 Materials and Methods

2.1 Source of seeds

Capsicum annuum var. 'Sweet banana' was used for this study. The seeds were purchased from a commercial seed supplier (Yates NZ Ltd; Auckland).

For comparison a second variety of *C. annuum* ('Yolo wonder') was also obtained from the same source.

2.2 Growth media

The media used in the study are listed below with the abbreviations used hereafter.

Germination medium (WA): 0.8% (w/v) Bacteriological agar (Germantown, New Zealand) in distilled water.

Murashige and Skoog medium (MS): Murashige and Skoog (1962).

Shoot induction medium (SIM): MS medium containing 5%(w/v) benzylaminopurine (BA) and 3%(w/v) sucrose.

Initiation medium (IM): SIM with 8%(w/v) agar

Luria broth (LB): (Appendix D)

Growth media was autoclaved, allowed to cool to room temperature and then the filter sterilized stock antibiotic solution was added to obtain the desired concentration. All operations requiring aseptic techniques were carried out in a Laminar flow cabinet.

2.3 *Capsicum* seed germination

Seeds were surface sterilized in 20%(v/v) household bleach containing 5%(v/v) sodium hypochlorite for 10 minutes and rinsed thoroughly 4 times with sterile distilled water. The media for seed germination was water agar(WA). The agar was dissolved

in distilled water by microwaving. 50 ml of molten agar was aliquoted in 200ml tissue culture pottels. These were autoclaved at 121°C, 15psi for 20 minutes. 12 surface sterilized seeds were placed in each pottel under aseptic conditions and kept at 25(±1)°C in continuous light, at approximately 100$\mu EM^{-2}s^{-1}$.

2.4 Age and source of explant

Explants used in the experiments were from aseptically germinated 11 day old seedlings. The seedling was cut into the following parts for *in vitro* culture cotyledon, upper hypocotyl (part of the hypocotyl close to the shoot apex), lower hypocotyl (part of the hypocotyl close to the root) and root explant (Plate 2).

Explants were obtained from 2,4,8,10 and 16 plants grown in the greenhouse. The plant material was surface sterilized in 20%(v/v) household bleach containing 5%(v/v) sodium hypochlorite for 10 minutes and rinsed thoroughly 4 times with sterile distilled water.

2.5 Preliminary experiments

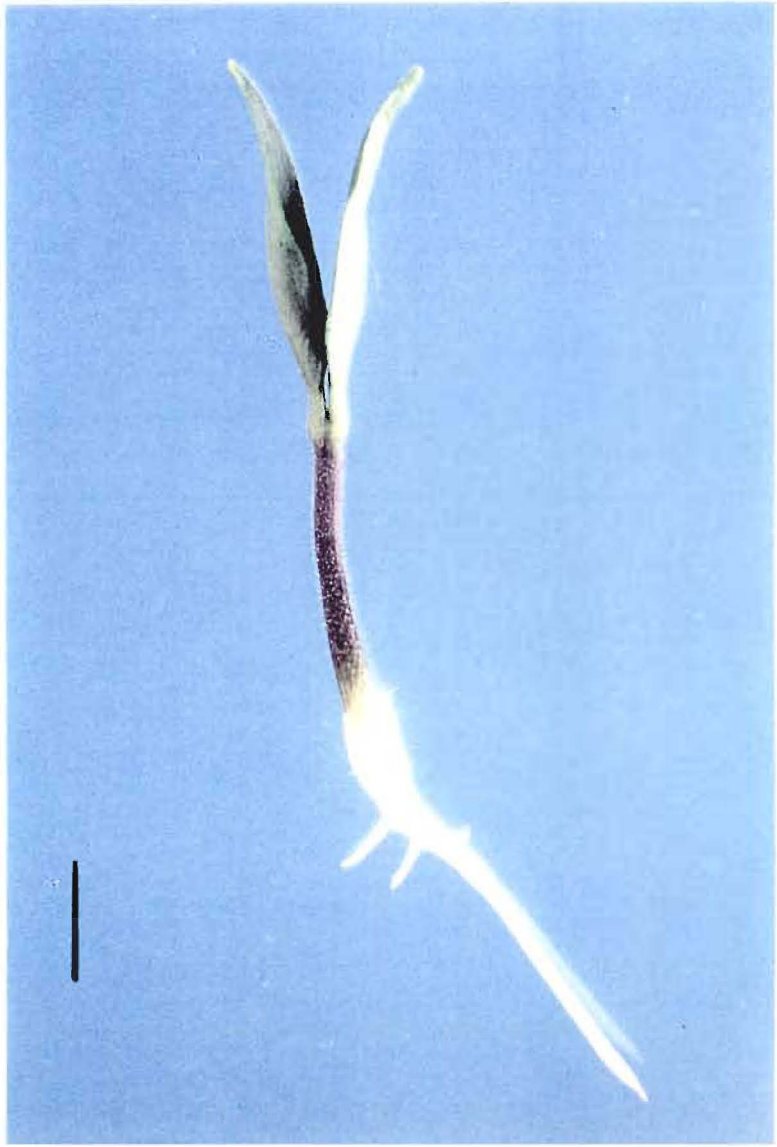
2.5.1 Shoot induction

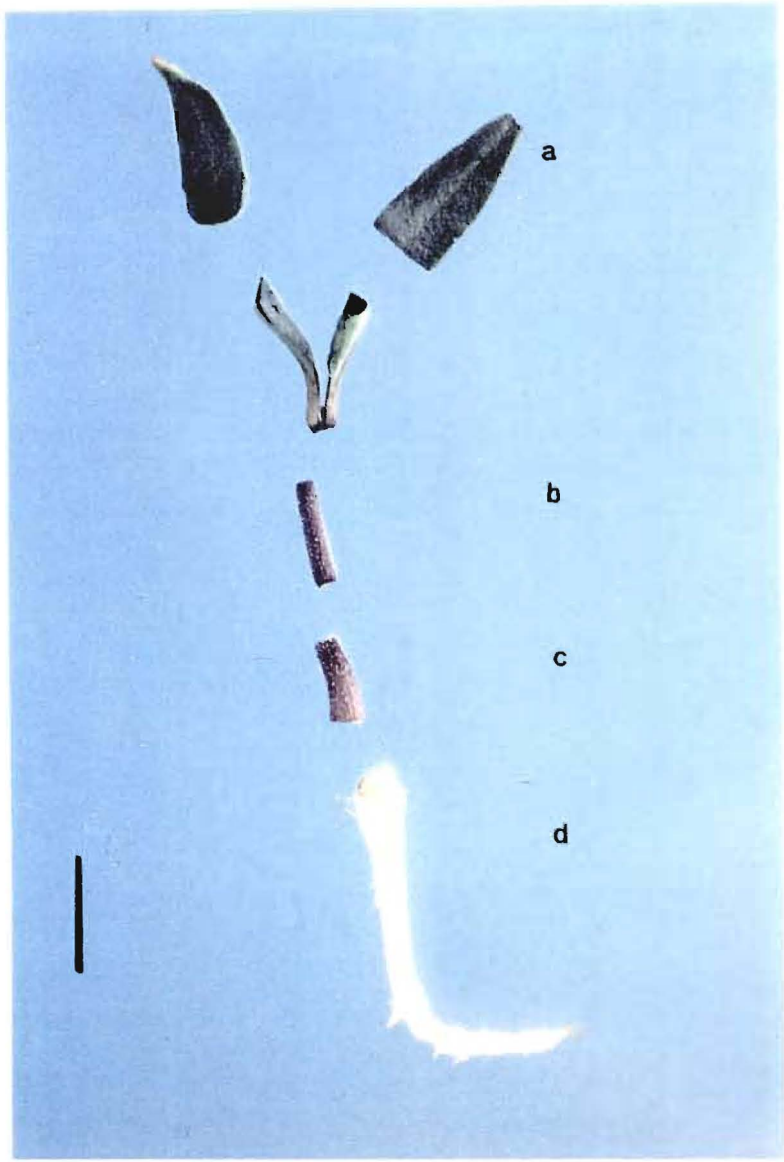
The upper hypocotyl was excised and cultured in sterile 20ml liquid shoot induction media (SIM) in 50ml pottels in a growth room at 25(±1)°C under continuous light for 20 days.

2.5.2 Sensitivity of explant to the antibiotic kanamycin

Sensitivity of the upper hypocotyl explant was tested at the following concentrations of kanamycin: $0mgL^{-1}$, $5mgL^{-1}$, $10mgL^{-1}$, $25mgL^{-1}$, $50mgL^{-1}$, $100mgL^{-1}$, $150mgL^{-1}$ and $200mgL^{-1}$. The explants were cultured in SIM containing kanamycin at these different concentrations. For each concentration there were twenty replicates. After 20 days the percentage of explants forming shoots was observed. In order to determine fresh weight change at these different kanamycin concentrations the

Plate 2. Axenically grown 11 day old *C.annuum* L. ('Sweet banana') seedling. Excised explants are shown as: **a**- cotyledons; **b**- upper hypocotyl; **c**-lower hypocotyl and **d**- root. (bar = 5mm)





upper hypocotyl was excised and cultured in SIM having kanamycin after recording the original fresh weight. After 20 days the final fresh weight was recorded.

2.5.3 Transfer from SIM+K to SIM

To determine whether the kanamycin inhibition of shoot induction of the upper hypocotyl was stage specific transfer experiments were carried out. The explant was cultured on SIM+K and transferred daily to SIM for 20 days. The proportion of explants forming shoots was recorded after 20 days.

The reverse transfer of upper hypocotyl from SIM to SIM+K was also carried out over a 20 day period.

2.5.4 Tolerance of explant to Claforan

The upper hypocotyl was excised and cultured in SIM containing 200mgL^{-1} Claforan for 20 days and the percentage of explants forming shoots was observed.

2.6 Establishment of regenerated shoots

Regenerated shoots from upper hypocotyl explants were excised and cultured on 20ml sterile culture media in 50ml tissue culture pottels. This media consisted of MS basal media, 0.8%(w/v) agar and 3%(w/v) sucrose. To enable shoot and root formation it was supplemented with varying concentrations of napthalene acetic acid and indole butyric acid. SIM containing 8%(w/v) agar was also used to establish new growth of shoots. After 4-6 weeks plants were transferred to half strength MS.

2.7 Bacterial strains and plasmids

For the purpose of genetically transforming *C. annuum* L. var 'Sweet banana' and 'Yolo Wonder' three strains of *A. tumefaciens* were used. These are listed in Table 1. The plasmid carrying the antibiotic resistance gene and GUS reporter gene (pBI 121; Jefferson *et al.*, 1987) was isolated from *E. coli* (Leung 1) and similarly the plasmid with the same antibiotic resistance gene and an intron containing GUS reporter

gene was isolated from *E.coli* (pIG 121; Ohta *et al.* 1990). Both these plasmids were mobilised into the three strains of *A. tumefaciens* used in the study.

2.7.1 Growth of *A. tumefaciens*

A single colony of untransformed *A. tumefaciens* (A4T, C58 and LBA4404) was inoculated into a 250ml capacity conical flask containing 50ml LB. Transformed strains of *A. tumefaciens* were inoculated into LB+50mgL^{-1} kanamycin. The cultures were incubated at 26(±1)°C and shaken at 200rpm.

2.7.2.Growth curve of *A. tumefaciens*

A. tumefaciens strains were grown (as described in 2.7.1.) and at 12, 18, 24, 30, 36, 42, 48 and 54 hours, 250µl of culture was removed and added to 750µl LB. Absorbance at 600nm was measured using a spectrophotometer (Unicam SP 1800 Ultraviolet Spectrophotometer). The absorbance reading of the sample was converted to cell density (Maniatis *et al.*, 1982) for each culture. Graphs were plotted to determine the growth phases of each strain.

2.8 Isolation of plasmid DNA

2.8.1 Alkaline Lysis method (adapted from Maniatis *et al.*, 1982)

This method is usually used for the isolation of plasmid DNA on a small scale. The DNA is not pure but it can be used for restriction enzyme digests and may be analysed by agarose gel electrophoresis. The amount of DNA isolated in this study was sufficient to transform *Agrobacterium* successfully.

An overnight culture of *E. coli* (harbouring the required plasmid) grown at 37°C in LB containing 50mgL^{-1} kanamycin was used. The plasmid DNA was isolated and its presence confirmed by determining the size of the cut fragment when the plasmid was digested with the restriction enzymes *Eco R1* and *Hind 111* for 1 hour at

Table 1: *A. tumefaciens* strains used in the study with the plasmid type found in each strain and the type of opine produced (Adapted from Dommisse *et al.*, 1990).

Strain	Plasmid type	Type of opine produced
C58	pTiC58	Nopaline
A4T	pRiA4	Agropine
LBA4404	pAL4404	none

(Strain A4T has the same chromosomal background as C58 but it contains an Ri plasmid.)

37°C in a total volume of 20µl. This was run on a 0.7%(w/v) agarose gel for 1 hour at 80 volts then stained with ethidium bromide for 30 minutes. After destaining, the gel was photographed under UV light.

The concentration and purity of the DNA in the preparation was determined by measuring the absorbance at 260nm and 280nm with a UV spectrophotometer (Unicam SP 1800 Ultraviolet Spectrophotometer).

2.8.2 Method for isolation of DNA from *Agrobacterium* (Slusarenko, 1990)

The rapid mini-prep method was used to isolate plasmid DNA and confirm the presence of the correct plasmid in each strain. The DNA obtained was analysed in the same manner as in 2.8.1.

2.9 Preparation of competent *A.tumefaciens* cells and transformation with plasmids pBI 121 and pIG 121

The three *A. tumefaciens* strains were transformed with either plasmid pBI 121 or plasmid pIG 121 using a modified freeze-thaw method of Höfgen and Willmitzer (1988).

The bacteria were grown in 5ml of LB at an incubation temperature of 26(±1)°C in a shaking water bath overnight. The culture was then diluted in 200ml LB and returned to the optimum temperature for 3-4 hours with shaking at 250rpm to enable aeration. Then the logarithimically growing cells were centrifuged at 3000g for 20 minutes. The pellet was washed in TE (tris-ethylenediaminotetra-acetic acid) and stored in LB in 500µl aliquots. 1µg of plasmid DNA was added to the thawed cells. The cells were then incubated successively for 5 minutes on ice, 5 minutes in liquid nitrogen and 5 minutes at 37°C. After dilution in 1ml LB the cells were grown at 28°C for 2-4 hours and plated out on selection media (LB+50mgL^{-1} kanamycin). Any resulting colonies were then reselected three times to ensure the plasmid was present conferring the resistance to the antibiotic and then placed in long-term storage (Maniatis *et al.* 1982). The resulting 6 new strains are shown in Table 2.

Table 2: A list of *A.tumefaciens* strains with the plasmids used in this research

C58	C58::pBI 121	C58::pIG 121
A4T	A4T::pBI 121	A4T::pIG 121
LBA 4404	LBA 4404::pBI 121	LBA 4404::pIG 121

2.10 Co-cultivation of *C. annuum* with *A.tumefaciens*

Outlined below is the general protocol used for the co-cultivation of *C. annuum* with *A. tumefaciens*. Modifications were made to the general protocol to vary the conditions for transformation. These are summarised in a series of tables (Table 3, 4, 5 and 6) and listed in Appendix G.

An overnight culture of an *A. tumefaciens* strain was grown in 50ml LB+50mgL^{-1} kanamycin. The culture was then centrifuged at 11 000g for 20 minutes at 20°C. The supernatant was discarded and the pellet was resuspended in 10ml of SIM.

The explant was inoculated with the resuspended culture and incubated for a defined period of time at 26(±1)°C in the dark. The control inoculum was SIM. Explants were transferred directly to selection medium, SIM with 50mgL^{-1} kanamycin and 200mgL^{-1} Claforan (SIM+K+C) for 20 days.

In some of the experiments the explant was first transferred to initiation medium (IM) with or without antibiotics for 24-72 hours. After this co-cultivation period, the explant was transferred to the selection medium SIM+K+C for 20 days.

To ascertain whether T-DNA transfer had been successful the histochemical assay for β-glucuronidase activity was carried out (2.11). In co-cultivation experiments using plasmid pIG 121 as the vector, selection was not necessary and the assay for β-glucuronidase activity was carried out after the period of co-cultivation.

2.11 Histochemical GUS assay

To confirm whether plant cells were expressing bacterial DNA (T-DNA), a histochemical assay was carried out.

The method was based on Jefferson *et al.* (1987). 10ml of the reaction buffer contained 50mM Sodium phosphate buffer (pH 7) and 5mg of X-GLUC (Sigma),(the substrate for the enzyme β-glucuronidase dissolved in 50µl dimethylformadide). 20% methanol was added (v/v) to the solution to eliminate endogenous GUS or GUS like activity (Koshugi *et al.*, 1990).

Each explant was placed in 200µl of GUS assay buffer in a well of a microtitre plate. The controls were explants that did not undergo transformation procedure. The microtitre plates were wrapped in Aluminium foil to prevent exposure to light and incubated in a water bath at 37°C. After 16 hours tissue was checked for blue precipitation indicative of transformation. The explants were then transferred to 80% ethanol and kept at 4°C to prevent further enzyme activity until explants could be photographed.

Table 3: Summary of co-cultivation experiments involving *C. annuum* L. ('Sweet banana') and *A. tumefaciens* with the vector pBI 121. An overnight culture of the appropriate strain was used as the inoculum and the control inoculum was SIM. The incubation temperature for the explants were incubated at 26(±1)°C.

Explant	*Agrobacterium* strain	Period of inoculation (minutes)	Period of co-cultivation	Selection medium (for 20 days)	Experiment number (Appendix G)
upper hypocotyl	C58::pBI 121	60	-	SIM+K+C	1 & 2
upper hypocotyl	C58::pBI 121	30	-	SIM+K+C	3
upper hypocotyl	C58::pBI 121	30	-	SIM+K+C	10
upper hypocotyl	C58::pBI 121	30	48 hours on IM	SIM+K+C	9
upper hypocotyl	C58::pBI 121	30	72 hours on IM or IM+K+C	SIM+K+C	8
upper hypocotyl	C58::pBI 121 (1)[a]	30	72 hours on IM or IM+K+C	SIM+K+C	4
upper hypocotyl	C58::pBI 121 (0.2)[b]	30	72 hours on IM or IM+K+C	SIM+K+C	4
upper hypocotyl	C58::pBI 121 (0.5)[c]	30	72 hours on IM or IM+K+C	SIM+K+C	4
upper hypocotyl	LBA4404::pBI 121	30	-	SIM+K+C	7

[a] overnight culture of *Agrobacterium tumefaciens* pelleted and resuspended in 10ml SIM
[b] 0.2 dilution of overnight culture that was pelleted and resuspended in 10ml SIM
[c] 0.5 dilution of overnight culture that was pelleted and resuspended in 10ml SIM

Table 4: Summary of co-cultivation experiments involving *C. annuum* L. ('Sweet banana') and *A.tumefaciens* with the vector pIG 121. The explants were inoculated for 60 minutes with an overnight culture of the appropriate strain. The control inoculum was SIM. The explants were incubated at 26(±1)°C .In the experiments shown below the upper hypocotyl of 11-day old seedling was pre-conditioned by culturing in SIM for a defined period of time.

Explant	*Agrobacterium* strain	Period of co-cultivation	Experiment number (Appendix G)
pre-conditioned for 4 days in SIM	all *	24 hours on IM+C	11
pre-conditioned for 4 days in SIM	all	48 hours on IM+C	12
pre-conditioned for 4 days in SIM	all	72 hours on IM+C	13
pre-conditioned for 4 days in SIM+K	all	48 hours on IM+C	19
pre-conditioned for 8 days in SIM	all	48 hours on IM+C	18
pre-conditioned for 0 days	all	48 hours on IM+C	20

* A4T::pIG 121, C58::pIG 121 and LBA4404::pIG 121

Table 5: Summary of co-cultivation experiments of axenically grown *Capsicum annuum* L. ('Sweet banana') 11-day old seedling and *A.tumefaciens* strains with the plasmid pIG 121. An overnight culture of the appropriate strain was used as the inoculum and the control inoculum was SIM. The explants were incubated at 26(±1)°C.

Explant	*Agrobacterium* strains	Period of incubation (minutes)	Period of co-cultivation	Experiment number (Appendix G)
cotyledon upper hypocotyl lower hypocotyl root	A4T::pIG 121	60	48 hours on IM+C	14
cotyledon upper hypocotyl lower hypocotyl root	C58::pIG 121	60	48 hours on IM+C	14
cotyledon upper hypocotyl lower hypocotyl root	LBA4404::pIG 121	60	48 hours on IM+C	14

Table 6: Summary of co-cultivation experiments with explants taken from young to mature *C. annuum* L. ('Sweet banana') plants with *A. tumefaciens*. An overnight culture of the appropriate strain was used to inoculate the explants for 60minutes. The control inoculum was SIM. The explants were incubated at 26(±1)°C .

Explant	*Agrobacterium* strains	Period of co-cultivation	Experiment number (Appendix G)
Mature plant (16 weeks): leaf segment	all*	48 hours on IM+C	15, 21, 22 & 23
Mature plant (16 weeks): stem sections	all	48 hours on IM+C	as above
Mature plant (16 weeks): petals and anther	all	48 hours on IM+C	as above
petals and anthers from 3 stages of flower formation	all	48 hours on IM+C	24
stem and leaf from 2 week old plant	all	48 hours on IM+C	17
stem and leaf from 4 week old plant	all	48 hours on IM+C	17
stem and leaf from 8 week old plant	all	48 hours on IM+C	17
stem and leaf from 10 week old plant	all	48 hours on IM+C	17

* A4T::pIG 121, C58::pIG 121 and LBA4404::pIG 121

CHAPTER 3

RESULTS

3 RESULTS

3.1 Preliminary experiments

The preliminary experiments were carried out to verify the shoot induction properties of the upper hypocotyl explant of axenically grown 11-day old seedlings of *Capsicum annuum* L. ('Sweet banana').

The upper hypocotyl was cultured for 20 days in 20ml SIM. It was a highly regenerative explant. At the end of the culture period 80-100% of explants formed shoot buds. The upper hypocotyl segment when excised from the seedling was 3-5mm in length and black in colour. After 4 days in the inductive medium (SIM) it loses its dark colouration and becomes light green. The explant elongates and swells. Between the 6th and 8th day of culture, the basipetal end of the upper hypocotyl was markedly more swollen than the acropetal end and wound callus is evident. By day 10 dark green 'spots' appear around the circumference of the cut ends. The green 'spots' are predominantly found on the basipetal end and these later become more visible as shoot primordia as they continue to develop further. At the end of 20 days a rosette of buds are formed (Plate 3).

3.2 Development of a selection stratergy and regeneration protocol

This section details the selection stratergy used for detecting kanamycin resistant upper hypocotyl transformants and the culture conditions developed to regenerate the transformed shoots.

Plate 3.

Upper hypocotyl explants:

A. After 4 days culture in SIM

B. After 20 days culture in SIM, shoot primordia have undergone further differentiation and distinct leafy shoots are formed.

(Bar = 1mm)

A

B

3.2.1 Percentage of upper hypocotyl explants forming shoots at different concentrations of kanamycin

The *Agrobacterium* strains used for the transformation experiments carried binary plasmids that had the selectable marker gene for Neomycin Phosphotransferase (NPT II). This confers resistance to the antibiotic kanamycin in cells that express the T-DNA.

In order to select explants that were kanamycin resistant after transformation the sensitivity of the explant to varying concentrations of kanamycin was first investigated. The upper hypocotyl of 11-day old axenically grown seedling of *C. annuum* L. ('Sweet banana') was excised and cultured in 20ml of SIM. Response to kanamycin was tested at eight different concentrations ($0mgL^{-1}$, $5mgL^{-1}$, 10mg L^{-1}, $25mgL^{-1}$, $50mgL^{-1}$, $100mgL^{-1}$, $150mgL^{-1}$ and $200mgL^{-1}$). The two aspects investigated were the effect kanamycin had on the explant to form shoots and the fresh weight change in the explant during culture in the presence of kanamycin.

Shoot formation was not effected by the presence of kanamycin in the medium at low concentrations ($0mgL^{-1}$ -$10mgL^{-1}$). At $25mgL^{-1}$, a marked decrease in the number of explants forming shoots was observed. Shoot formation was completely inhibited for kanamycin concentrations of $50mgL^{-1}$, $100mgL^{-1}$, $150mgL^{-1}$ and $200mgL^{-1}$ (Table 7).

The percentage of explants forming a rosette of shoot buds after 20 days culture in SIM (without kanamycin) was 85%. At $50mgL^{-1}$ the explant had retained its green colouration and undergone elongation and swelling whereas at $100mgL^{-1}$ kanamycin the explant was less green in appearance and not as elongated. Both showed no evidence of green 'spots 'or shoot bud formation (Plate 4). For the purpose of selection of transgenic tissue, $50mgL^{-1}$ kanamycin was chosen as the most appropriate concentration for these reasons.

Table 7

Percentage of upper hypocotyl explants forming shoots when cultured in SIM containing different concentrations of kanamycin for 20 days.

Concentration of kanamycin in SIM (mgL^{-1})	Percentage of upper hypocotyls forming shoots
0	85[a]
5	80
10	85
25	40
50	0
100	0
150	0
200	0

[a] There were 20 explants for each treatment.

Plate 4.

Effect of different concentrations of kanamycin on shoot formation of upper hypocotyl explants:

A. After 20 days culture in SIM+0mg L^{-1} kanamycin

B. After 20 days culture in SIM+50mg L^{-1} kanamycin

C. After 20 days culture in SIM+100mg L^{-1} kanamycin

(bar = 1cm)

3.2.2 Fresh weight change of upper hypocotyl at different concentrations of kanamycin

The average fresh weight change in the upper hypocotyl explant after 20 days culture in SIM with 8 different concentrations of kanamycin ($0mgL^{-1}$, $5mgL^{-1}$, $10mgL^{-1}$, $25mgL^{-1}$, $50mgL^{-1}$, $100mgL^{-1}$, $150mgL^{-1}$ and $200mgL^{-1}$) was determined by weighing explants before and after culture. The results are summarised in Figure 2. Explants cultured in SIM with kanamycin concentrations of 50mg L^{-1} or higher showed a dramatic decrease in fresh weight change.

3.2.3 Transfer of explant from SIM+Kanamycin to SIM

Shoot formation was repressed in the presence of kanamycin at a concentration of $50mgL^{-1}$ or higher (3.2.1). To investigate whether the effect of kanamycin on shoot induction was specific to a particular stage of shoot induction and shoot formation the explant was cultured in SIM with $50mgL^{-1}$ kanamycin (SIM+K) and transferred daily to SIM (without kanamycin).

The percentage of upper hypocotyl explants that formed shoots when cultured in SIM was 80% and this declines to 60% when the explant has been in SIM+K for 4 days. When transferred on the 5th day, only 15% formed shoots. This trend continued until the 9th day. After 10 days in SIM+K shoot formation was completely inhibited (Table 8).

Figure 2: Fresh weight change of upper hypocotyl explant in different concentrations of kanamycin after 20 days

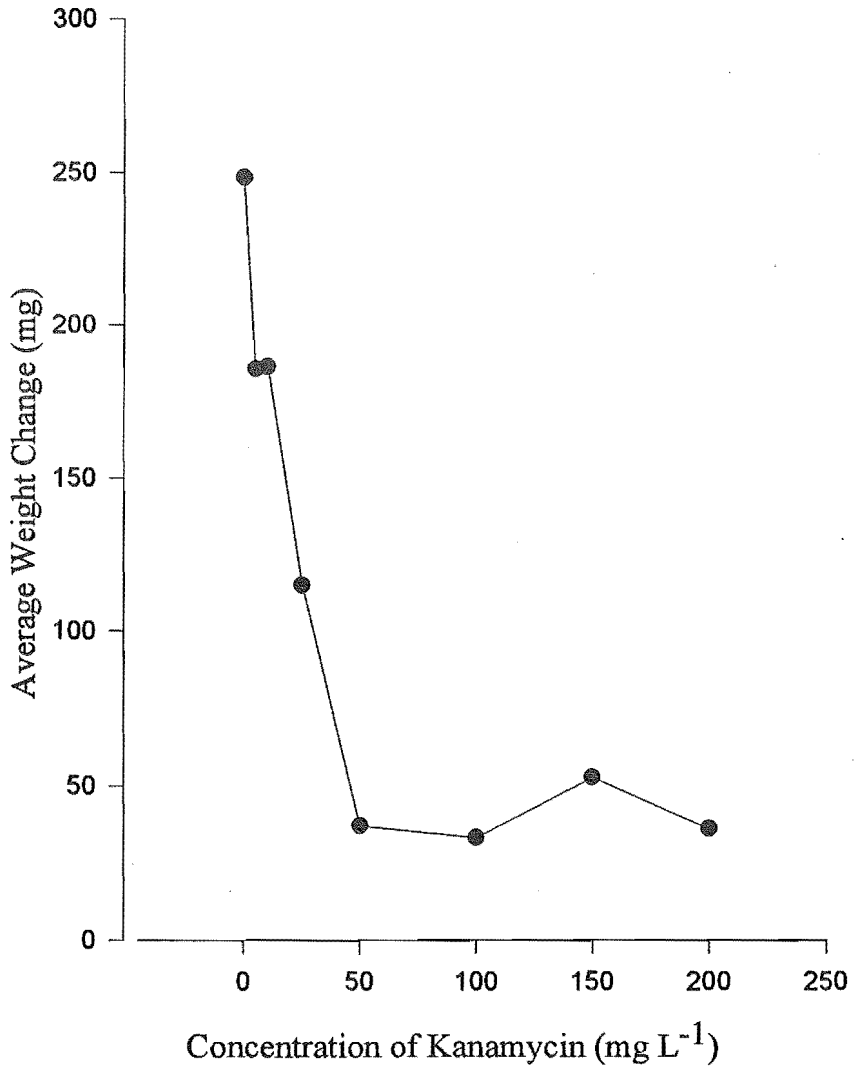


Table 8

Percentage of upper hypocotyl explants forming shoots when cultured on SIM Containing 50mgL^{-1} kanamycin (SIM+Kan) and transferred to SIM (without kanamycin).

Number of days in SIM+Kan	Percentage of hypocotyls forming shoots	
	Experiment 1	Experiment 2
0	80	80
1	90	80
2	80	60
3	80	80
4	60	60
5	20	10
6	10	20
7	10	10
8	0	20
9	0	10
10	0	0
11	0	0
12	0	0
13	0	0
14	0	0
15	0	0
16	0	0
17	0	0
18	0	0
19	0	0
20	0	0

Table 9:

Percentage of upper hypocotyl explants forming shoots when cultured on SIM (without kanamycin) and transferred to SIM containing 50mgL^{-1} kanamycin (SIM+Kan)

Day of transfer from SIM to SIM+Kan	Percentage of explant forming shoots
0	0
1	0
2	0
3	0
4	10
5	10
6	0
7	20
8	10
9	50
10	70

The reverse of the transfer experiment was carried out to determine whether, after shoot induction of the upper hypocotyl had been induced, kanamycin would affect the shoot formation ability. Upper hypocotyl explants were cultured in SIM and then transferred to SIM+K daily. The results showed that after 4 days in SIM transfer to SIM+K does not interfere with the shoot induction process (Table 9). These results complement the findings of the SIM+K to SIM transfer experiment. Kanamycin inhibited shoot induction if it was present in the medium for the first 4 days. Explants transferred from SIM to SIM+K formed shoots when the transfer was carried out before the 4th day. When transferred to SIM+K on the 4th day a low percentage of explants retained the ability to form shoots.

3.2.4 Tolerance of upper hypocotyl explant to Claforan

In the *Agrobacterium*-mediated transformation experiments, explants were maintained in culture with the second antibiotic Claforan. This antibiotic in the culture medium prevents the proliferation of *Agrobacterium* that contaminate the explant. The upper hypocotyl explant was tolerant to Claforan at 200mgL^{-1} (Table 10). There was a decrease in the percentage of explants that formed shoots, however *Agrobacterium* cells had to be prevented from growing in the shoot inductive medium. Observations from Section 3.4.1 confirmed that 200mgL^{-1} Claforan was sufficient to inhibit bactereial growth and this evident from Plate 8 showing explant in bacteria-free culture medium.

3.2.5 Regeneration of plants from shoot buds

Sections of the rosette of shoot buds formed on the upper hypocotyl were excised and subcultured on solid MS medium supplemented with various combinations of IBA and NAA. Development of the shoots, callus and root formation was assessed for a number of combinations of IBA and NAA (Appendix F).

Table 10

Percentage of upper hypocotyl explants forming shoots in SIM having Claforan ($200mgL^{-1}$) and kanamycin ($50mgL^{-1}$) after 20 days.

Medium	Percentage of explants forming shoots	
	Experiment 1	Experiment 2
SIM	80	80
SIM+C	60	60
SIM+K	0	0
SIM+K+C	0	0

The excised shoot buds produced roots and the shoot buds remained green after 4-6 weeks but did not develop into leaf structures when the medium was supplemented with 0.02 or $0.5mgL^{-1}$ IBA and $0.05mgL^{-1}$ NAA.

Shoot buds that were subcultured on solid MS medium without the exogenously supplied hormones continued to grow and develop leaf-like structures. These were then transferred after 4 weeks to half strength solid MS medium. Of the subcultured shoots that developed further and formed larger leaves root formation was not evident. The plants that survived a further subculture are shown on Plate 5. There was no attempt made to further investigate this section of the research.

3.3 Bacterial strains and plasmids

3.3.1 Isolation and confirmation of plasmids pBI 121 and pIG 121

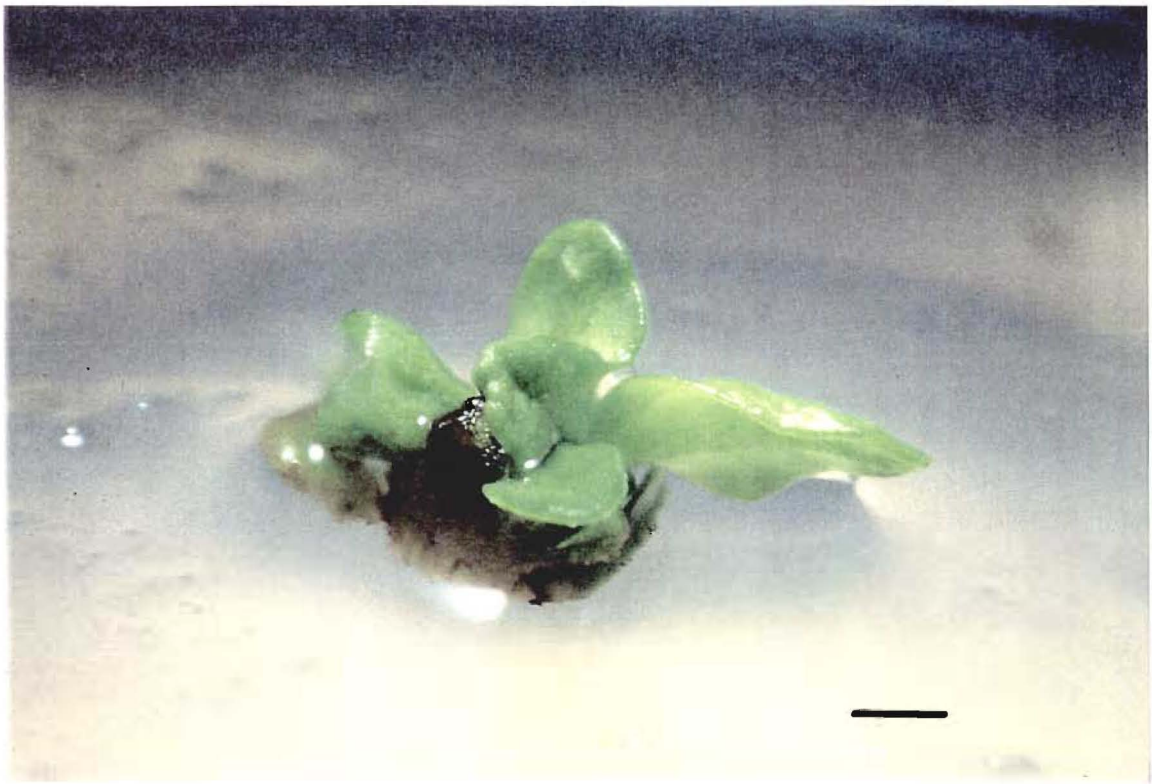
Plate 6 shows a photograph of plasmid DNA cleaved by restriction enzymes into fragments and seperated by agarose gel (0.7% w/v) electrophoresis. The plasmid DNA was isolated from *E. coli.* To confirm that the correct plasmid was isolated, its size and restriction sites were analysed. Plasmid pBI 121 is 13kbp and pIG 121 has 190bp more. Both have unique restriction sites for *Eco RI* and *Hind III* . Restriction enzyme digest with *Eco RI* and *Hind III* cuts the plasmid into two segments of 10kbp and the 3kbp GUS cassette.

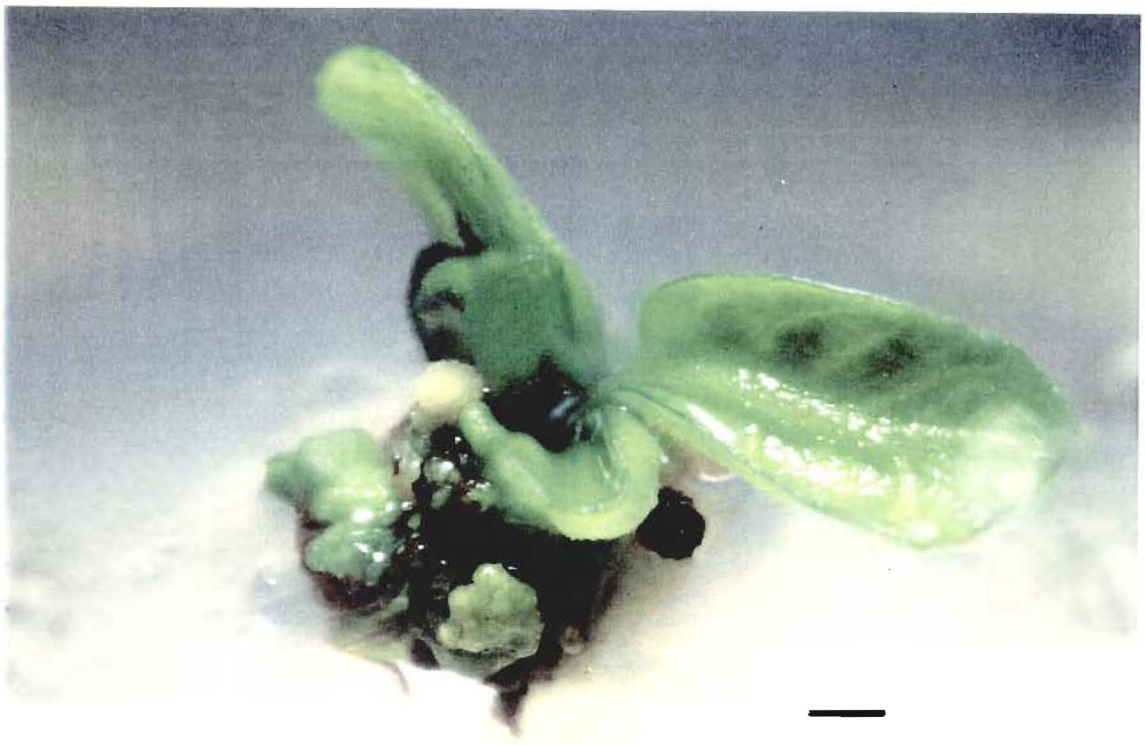
3.3.2 Growth curves of *Agrobacterium* strains

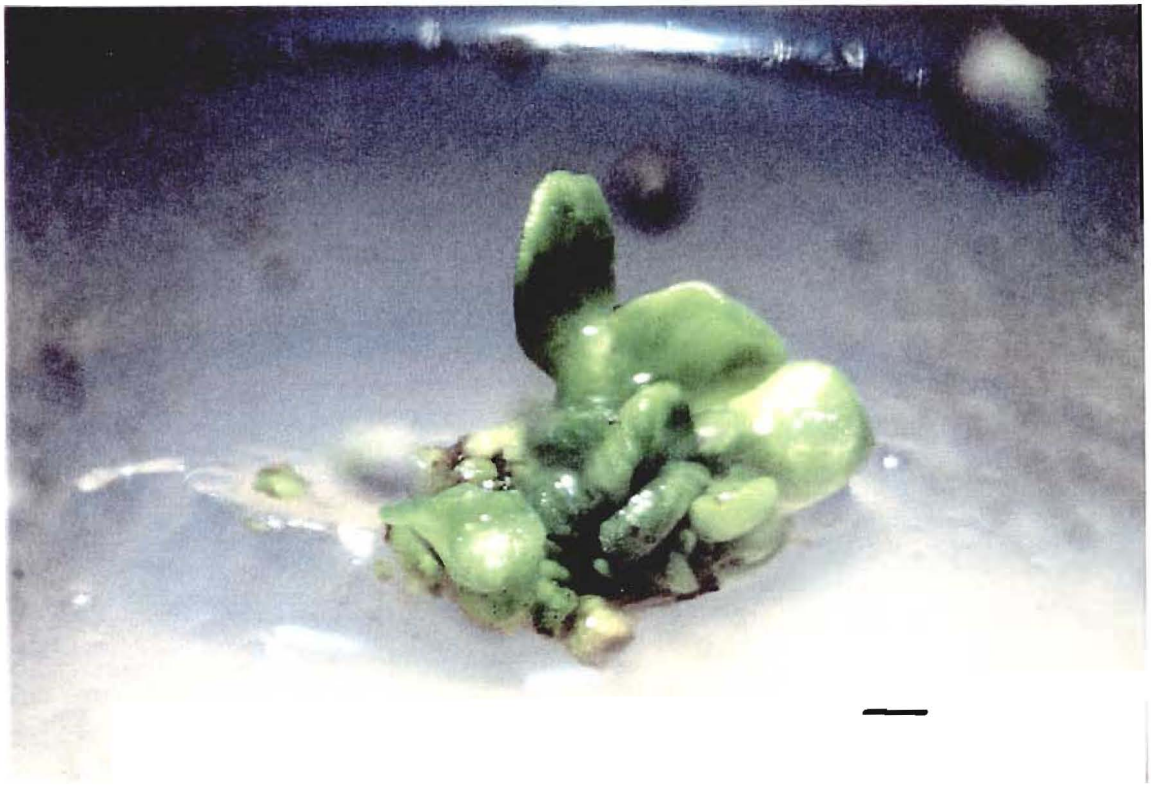
Figures 3, 4 and 5 show the growth curves of the *A. tumefaciens* in liquid culture. The growth curve was monitored to evaluate the growth phase of the bacteria strains as this maybe an important factor for the transformation of plants. The graphs show that between 12 and 20 hours the cells are undergoing exponential growth phase.

Plate 5.

Regenerated shoots from upper hypocotyl explants subcultured on solid MS medium. The shoot buds have developed into leaf-like structures.
The photographs were taken after 8 weeks growth.
(bar = 2mm)







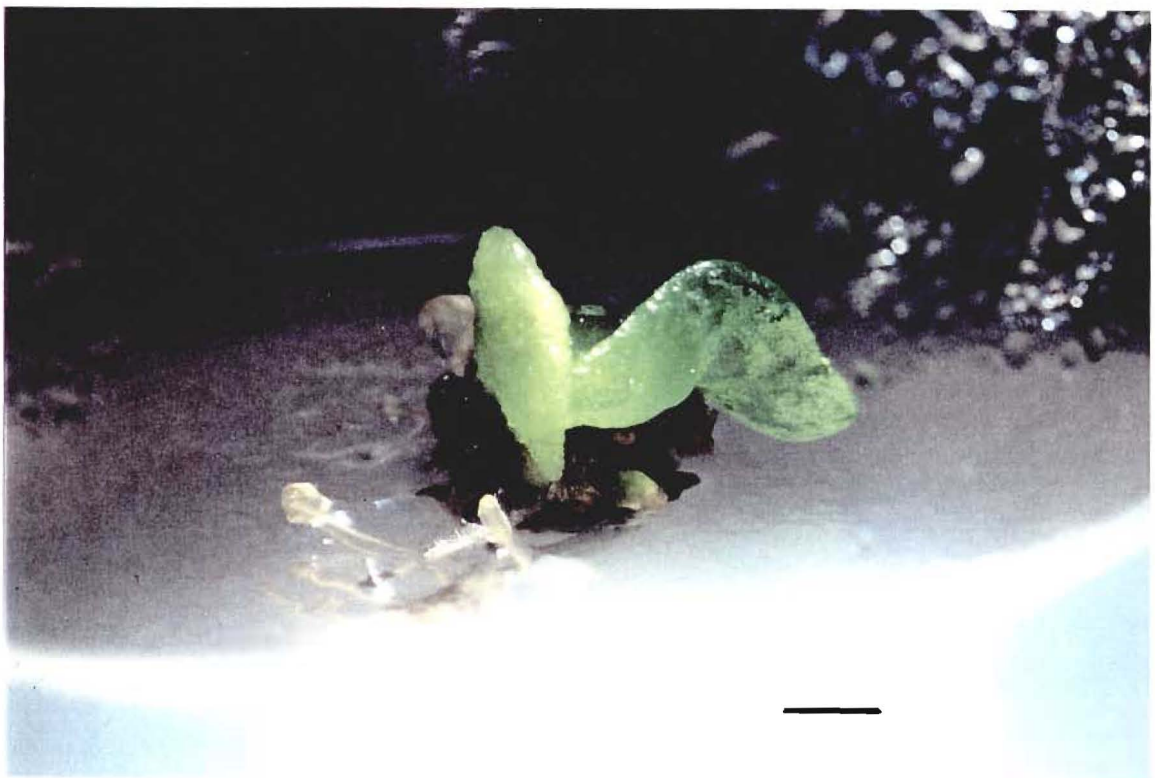


Plate 6.

Gel of plasmid pBI 121 and pIG 121 preparation

A. This lane contains λ*Hind III* molecular weight markers running from the top of the gel to the bottom as follows: 23.7kbp, 9.5kbp, 6.8kbp, 4.3kbp, 2.3kbp, 2.0kbp

B. Uncut pBI 121

C. pBI 121 double digested with *Eco RI* and *Hind III*

D. Uncut pIG 121

E. pIG 121 double digested with *Eco RI* and *Hind III*

Figure 3: Growth Curve of *A. tumefaciens* Strains A4T, A4T::pBI 121 and A4T::pIG 121

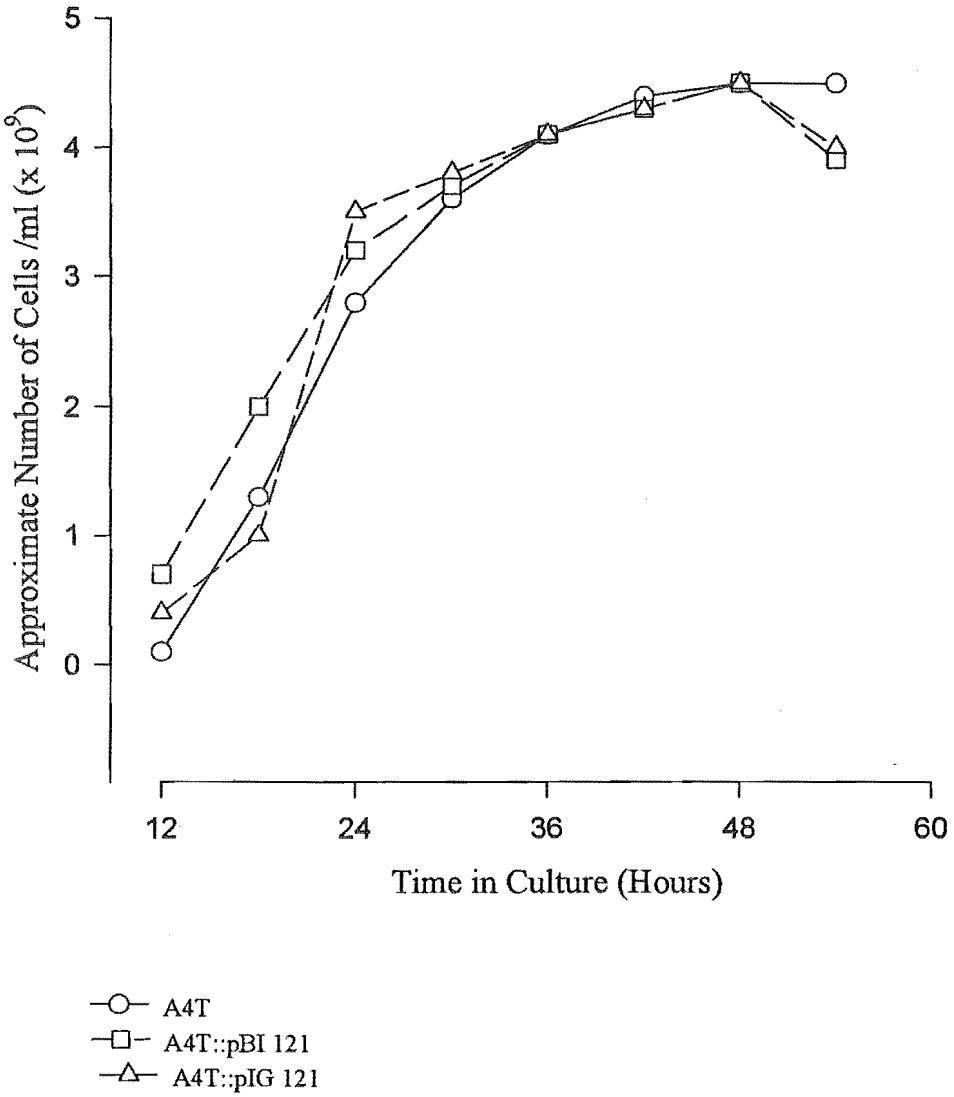


Figure 4: Growth Curve of *A. tumefaciens* Strains C58, C58::pBI 121 and C58::pIG 121

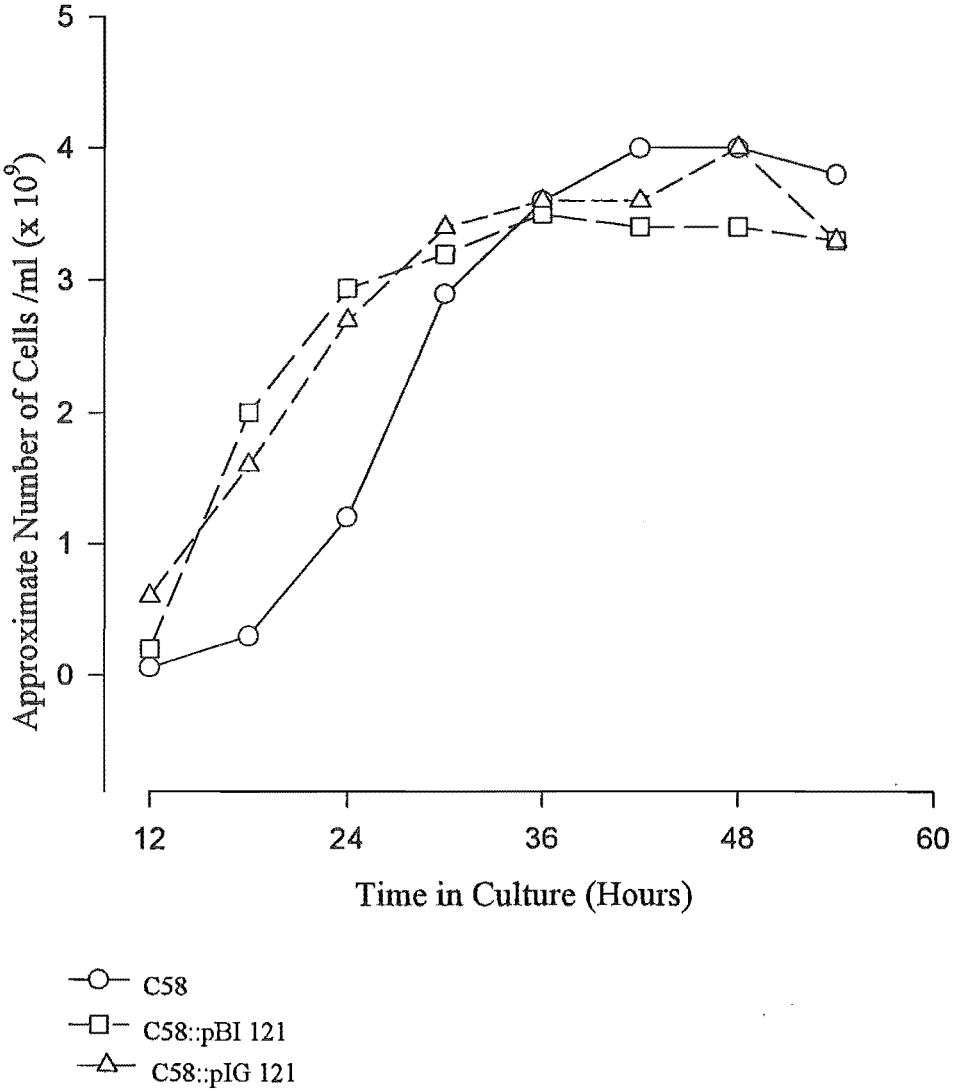
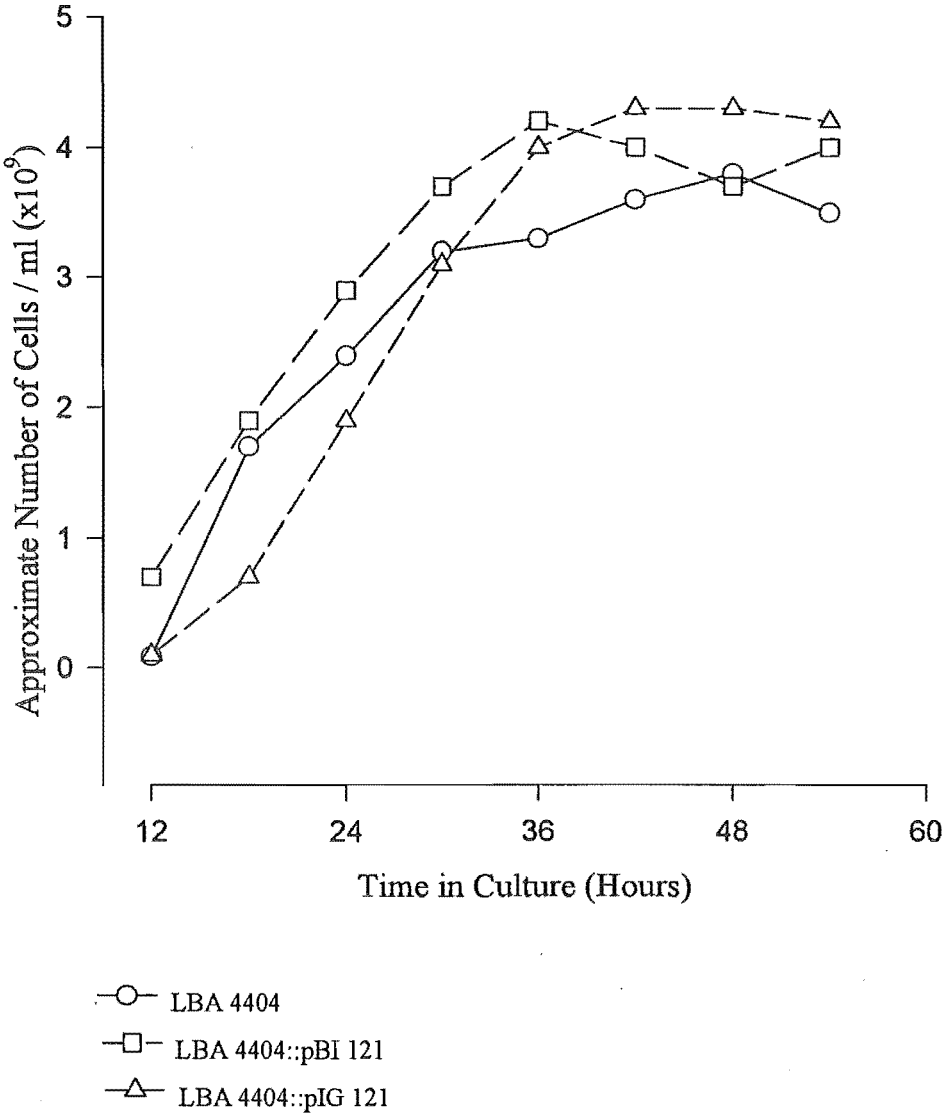


Figure 5: Growth Curve of *A. tumefaciens* Strains LBA 4404, LBA 4404::pBI 121 and LBA 4404::pIG 121



3.4 Transformation of *Capsicum* L. ('Sweet banana') with *A. tumefaciens*

The co-cultivation experiments that were carried out are listed as Experiments 1-23 (in Appendix G; the numbers do not reflect a chronological sequence). The results are summarised in Tables 11-14.

3.4.1 Co-cultivation experiments with *A. tumefaciens* strains having the binary plasmid pBI 121

In experiment 1,2 and 10 the upper hypocotyl was inoculated with the *A. tumefaciens* strain C58::pBI 121 for 30-60 minutes. The upper hypocotyl explants were then blotted dry and transferred to 20ml SIM+K+C for 20 days. None of the explants formed shoots. The outcome was the same in experiment 3 where the explants were inoculated with LBA 4404::pBI 121

To determine whether shoot formation was affected by the co-cultivation procedure a second control was added in Experiment 3. The explants were inoculated with SIM (control inoculum) and transferred to SIM rather than SIM+K+C for 20 days. From 10 explants, 6 formed shoots, indicating that the co-cultivation procedure was not detrimental to shoot formation.

Modificatiions were made to the transformation protocol in order to increase the time period for the *Agrobacterium* to interact with the plant cells, without submerging the explant in bactera. This was made possible by incubating the explant after inoculation with the *Agrobacterium* on IM (solid SIM) for 42-72 hours. The *Agrobacterium* that adhered to the explant were in contact with the explant hence increasing the chances for virulence induction and T-DNA transfer to occur. Another variable was to reduce the selection pressure by not including the antibiotics kanamycin and Claforan in IM.

The cells on the cut edge of the explants may also have been overcome by the concentration of bacterial cells in the inoculum. Hence the cell density of the inoculum was varied. The original number of cells in the overnight culture was pelleted and resuspended in 10ml of SIM. This was diluted to decrease the cell density.

In experiment 8 after inoculation with C58::pBI 121 the explants were incubated on either IM (without antibiotics) or IM+K+C for 72 hours and then transferred to 20ml SIM+K+C for 20 days. From 60 explants inoculated, 7 formed shoots whilst the control explants did not. The shoots appeared to be kanamycin resistant. To confirm the presence of T-DNA, the shoots were excised and assayed for GUS expression. The shoot buds proved negative for GUS assay.

Plate 7 shows a control upper hypocotyl after 20 days culture in SIM+K+C. As expected shoot formation was inhibited in untransformed explants. Plate 8 shows examples of explants with shoots formed after co-cultivation and selection but proved to be GUS negative. A closer examination of the pattern of shoot bud formation in these cases, when compared to the usual shoot bud rosette formed, may yield an explanation for the 'escapes'.

Experiment 4 was a repeat of Experiment 8 but here the control explant also formed shoot buds after culture for 20 days in SIM+K+C. The frequency of escapes was low but this confirmed that selection was not rigorous enough when the explants were not transferred directly to liquid SIM+K+C after inoculation. The effect of varying the cell density of the inoculum could not be evaluated as the results were negative.

In Experiment 9, explants were inoculated with C58::pBI 121 and treated as in Experiments 4 and 8. From 10 control explants 1 produced shoot buds and from 60

explants inoculated, 17 formed shoots that did not show GUS activity. Co-cultivation with A4T::pBI 121 (Experiment 5) and LBA4404::pBI 121 (Experiment 7) for 72 hours gave negative results.

In the experiments described above, exposure to kanamycin for selection was delayed by 72 hours. Incubating on IM+K+C did not have the ssame effect as selection in SIM+K+C. Since this delay in selection maybe the reason for control explants forming shoots the co-cultivation period was decreased to 48 hours (Experiment 6). Shoot formation was not observed in any of the explants and a repeat of this experiment was also not successful in obtaining transformants.

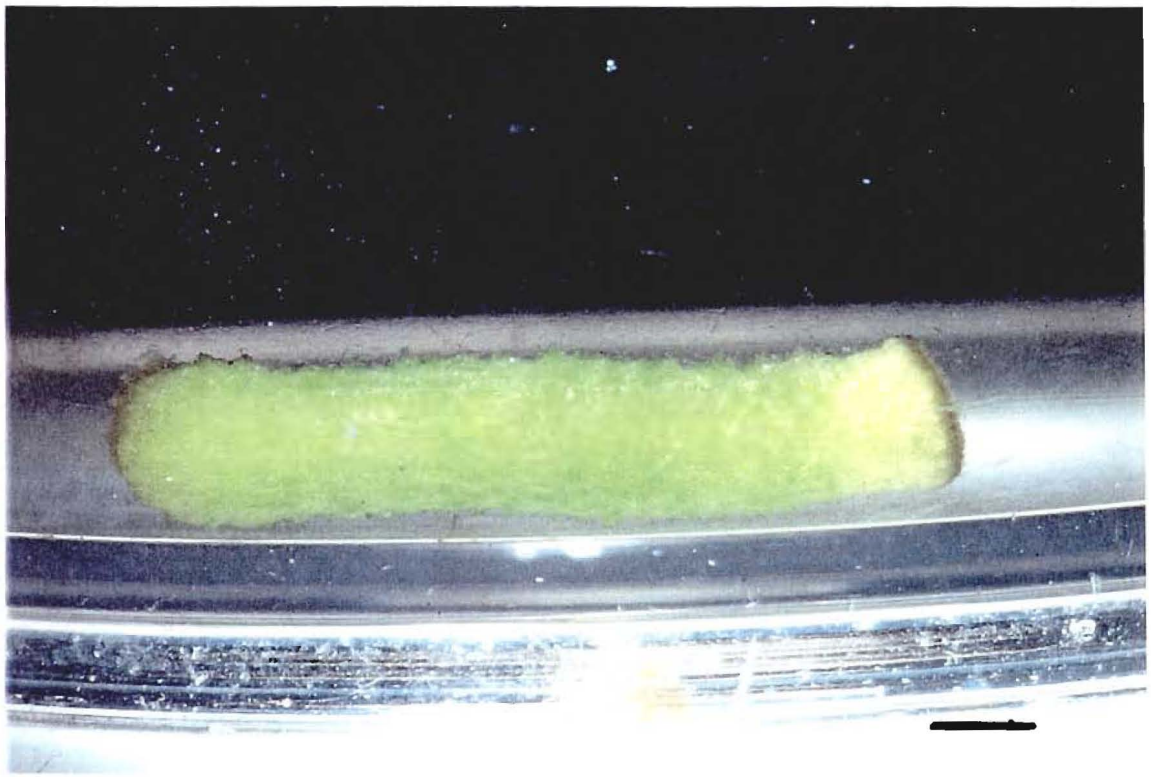


Plate 7.

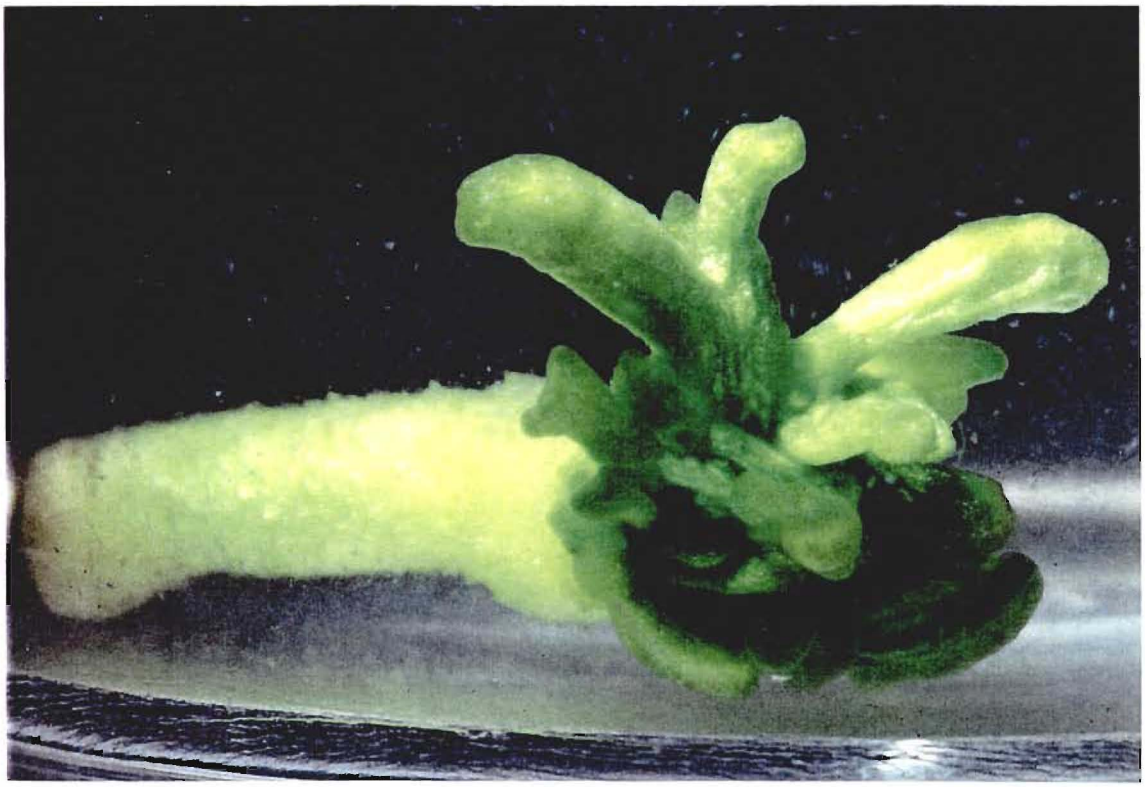
Upper hypocotyl cultured in SIM+K+C for 20 days

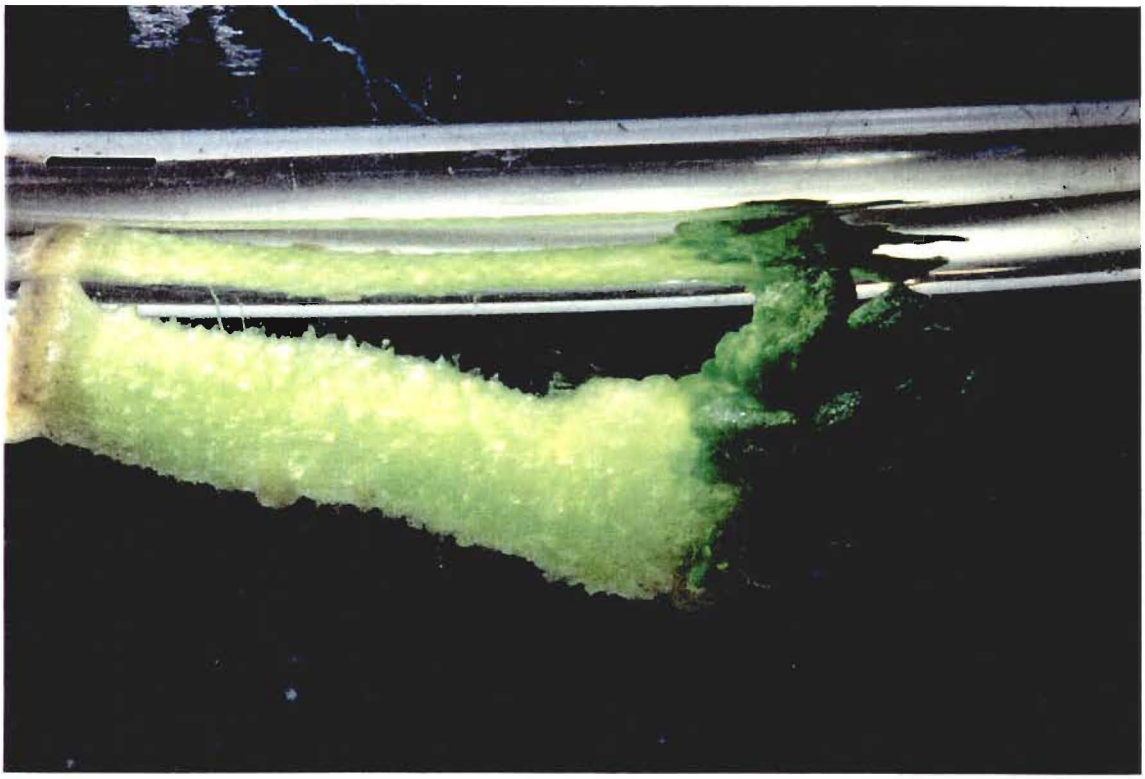
(bar = 2mm)

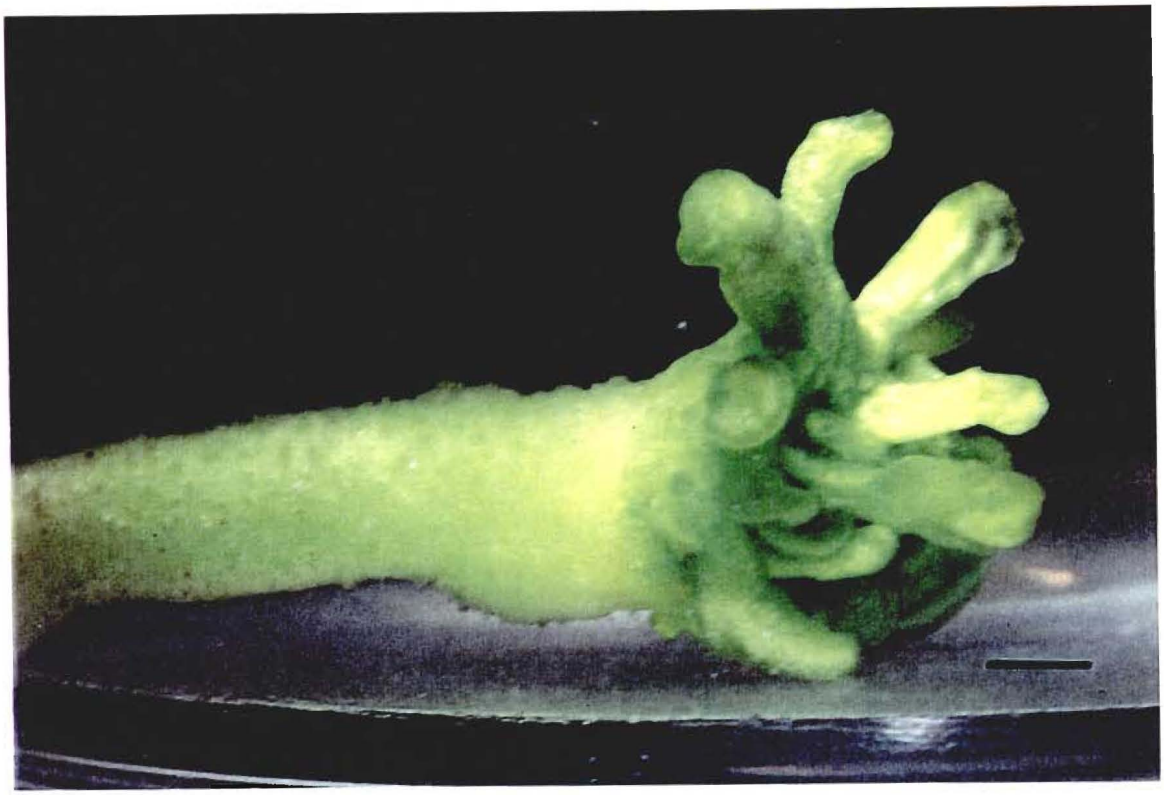
Plate 8.

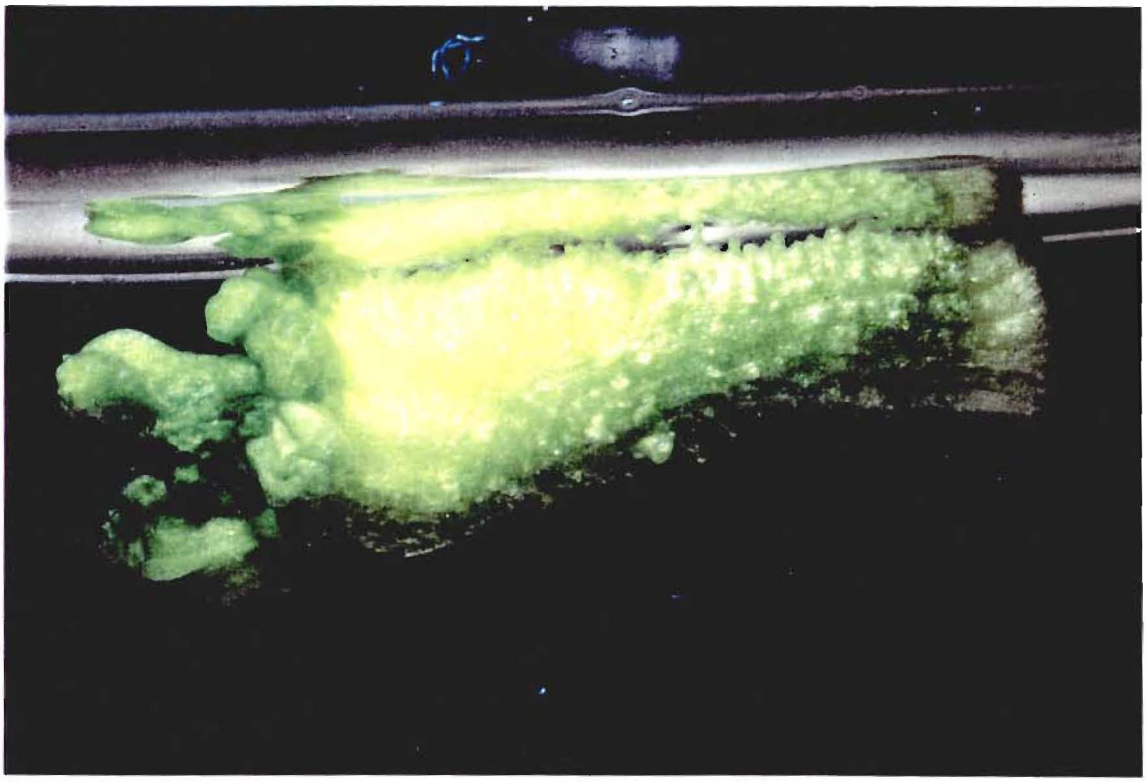
Upper hypocotyl explants that formed shoots after co-cultivation and selection protocol. However these shoots were GUS negative.

(bar = 2mm)









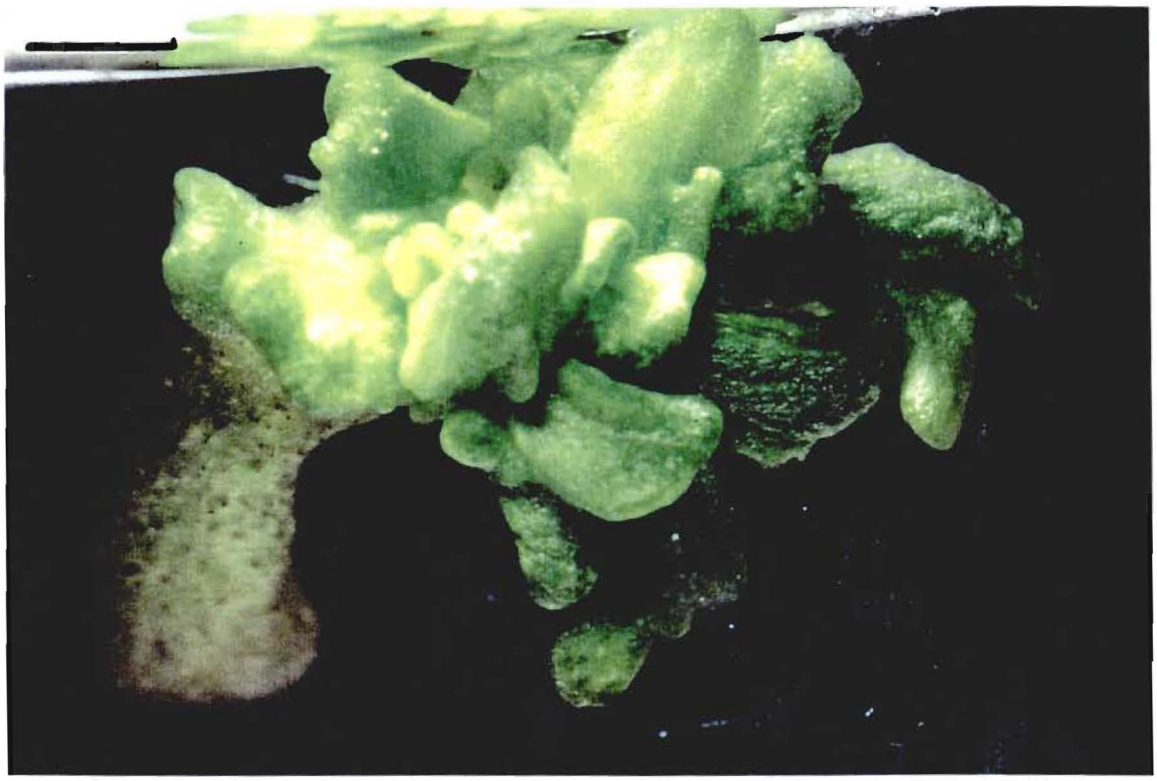


Table 11 Summary of results from co-cultivation experiments with *A. tumefaciens* having binary plasmid pBI 121

Explant	*Agrobacterium* strain	Inoculation (minutes)	Co-ultivation (hours)	Selection medium	Shoot formation	GUS staining	Experiment (Appendix G)
upper hypocotyl	control C58 :pBI 121	60	-	SIM+K+C for 20 days	no no	-	1 & 2
upper hypocotyl	control LBA4404pBI121	30	-	SIM+K+C for 20 days	no no	-	3
upper hypocotyl	control C58 :pBI 121	30	-	SIM+K+C for 20 days	no no	-	10
upper hypocotyl	control C58::pBI 121	30	72 on IM	SIM+K+C for 20 days	yes (10%) yes (28%)	negative negative	9
upper hypocotyl	control C58 :pBI 121	30	72 on IM or IM+K+C	SIM+K+C for 20 days	no yes	negative	8
upper hypocotyl	control C58::pBI121(1)[a] (0.2)[b] or (0.5)[c]	30	72 on IM or IM+K+C	SIM+K+C for 20 days	yes (control) yes	negative negative	4
upper hypocotyl	C58 :pBI 121	30	48 on IM+K+C	SIM+K+C for 20 days	no no	- -	6

[a] overnight culture of *A. tumefaciens* pelleted and resuspended in 10ml SIM
[b] 0.2 dilution of overnight culture that was pelleted and resuspended in 10ml SIM
[c] 0.5 dilution of overnight culture that was pelleted and resuspended in 10ml SIM

3.4.2 Co-cultivation experiments with *A. tumefaciens* strains having the binary plasmid pIG 121

The *A. tumefaciens* strains with the binary vector pIG 121 were used in the following experiments. The results are summarised in Tables 12, and 13. Selection of the antibiotic resistance marker was not required as the expression of β-glucuronidase enzyme activity could be directly assayed after co-cultivation. Expression was only possible if the T-DNA had been integrated in the plant genome and the intron within the GUS gene was spliced out by the plant eukaryotic gene expression system.

As is evident from Table 12 cotyledons, lower hypocotyl, upper hypocotyl and roots of 11 day old seedling were negative for GUS expression when co-cultivated for 48 hours (Experiment 14 and 20).

The results of the kanamycin sensitivity experiment (Section3.2.3) suggest that the first 4 days of culture in SIM are critical to the shoot induction process. Upper hypocotyl explants were therefore cultured in SIM for 4 days to allow the explant to undergo the development process that leads to shoot formation and then co-cultivated with *A. tumefaciens* strains for 24, 48 or 72 hours (Experiment 11, 12 and 13 respectively; Table 13). Since none of the explants expressed GUS. The time period for co-cultivation and the competence for T-DNA uptake of pre-conditioned explants could not be evaluated.

In experiment 19, upper hypocotyl explants were pre-conditioned for 4 days in SIM+K. Kanamycin inhibits shoot induction after 4 days culture in this medium. This experiment was to complement experiment 12. GUS was not expressed in the upper hypocotyls.

As in experiment 12, upper hypocotyl explants were pre-conditioned for 8 days in SIM (Experiment 18). At this stage in shoot induction and development the upper hypocotyl is on the verge of producing the dark green 'spots' (shoot primordia). The explants were co-cultivated with *Agrobacterium* for 48 hours. Explants were negative for GUS staining.

3.4.3 *Agrobacterium*-mediated transformation of mature tissue

C. annuum L. ('Sweet banana')

Co-cultivation experiments were carried out with leaf segments, stem sections, petals and anthers taken from 4 month old plants of *C. annuum* L. ('Sweet banana'). The explants were inoculated with *A. tumefaciens* strains A4T::pIG 121, LBA4404::pIG 121 and C58::pIG 121 and for 60 minutes and incubated on IM+C for 48 hours.

Leaf segments and stem sections expressed GUS activity (Experiment 15 and 21). The blue precipitation as a result of the GUS activity was evident in the leaf sections as spots (Plate 9; A) or present in the veins (Plate 9; B). Stem sections co-cultivated with C58::pIG 121 show GUS activity throughout the tissue with deeper staining in the vascular tissue (Plate 9; D, E & F). When co-cultivated with LBA4404::pIG 121 GUS activity was more evident around the edges of the vacular tissue (Plate 9; G & H).

Co-cultivation of flowers with C58::pIG 121 for 48 hours (Experiments 15, 21 & 22) resulted in GUS expression throughout the anther (Plate 9; J) and the petals expressed GUS around the edges and the cut base of the flower (Plate 9; K). Explants did not show GUS activity after co-cultivation with A4T::pIG 121. Endogenous GUS activity was not present in the tissue as control explants were free of blue colouration after the GUS assay.

To determine whether plants younger than 4 months were competent for transformation, explants were tested from 2, 4, 8 and 10 weeks old plant. There was no GUS expression in these tissues after co-cultivation (Experiment 17; Table 14).

Flowers at 3 different development stages were co-cultivated with *Agrobacterium* and assayed for GUS expression. This was to determine whether competency for transformation of the petal and anther explants varied with developmental stage of the flower (Experiment 23). The GUS assay results were negative for all explants tested.

On occassions the GUS assay was negative for explants that had been shown by previous trials to be competent for *Agrobacterium*-mediated transformation. This may have been due to the loss of the plasmid carrying the marker genes rather than the plant cells' lack of transformation competency.

3.4.4 *Agrobacterium*-mediated transformation of flowers of *C. annuum* L. ('Yolo Wonder')

'Yolo Wonder' petals and anthers expressed GUS after co-cultivation with *A. tumefaciens* strains C58::pIG 121 and LBA4404::pIG 121(Experiment 16). GUS activity was localised in the vein structures of the petals and around the edges of the anther (Plate 10).

Table 12

Summary of results from co-cultivation experiments involving *A. tumefaciens* and explants from 11-day old *C. annuum* L. ('Sweet banana') seedling

Explant	*Agrobacterium* strain	Period of inoculation (minutes)	Period of co-cultivation	GUS staining	Experiment number (Appendix G)
cotyledon upper hypocotyl lower hypocotyl root	A4T::pIG 121	60	48 hours on IM+C	negative	14
cotyledon upper hypocotyl lower hypocotyl root	C58::pIG 121	60	48 hours on IM+C	negative	14
cotyledon upper hypocotyl lower hypocotyl root	LBA4404::pIG 121	60	48 hours on IM+C	negative	14

Table 13

Summary of results from co-cultivation experiments using *A. tumefaciens* having binary plasmid pIG 121

Explant: upper hypocotyl	Strains	Inoculation (minutes)	Co-cultivation (hours)	GUS staining	Experiment number (Appendix G)
pre-conditioned for 4 days in SIM	control all*	60	24 on IM+C	negative	11
pre-conditioned for 4 days in SIM	control all	60	48 on IM+C	negative	12
pre-conditioned for 4 days in SIM	control all	60	72 on IM+C	negative	13
pre-conditioned for 4 days in SIM+K	control all	60	48 on IM+C	negative	19
pre-conditioned for 8 days in SIM	control all	60	48 on IM+C	negative	18
pre-conditioned for 0 days	control all	60	48 on IM+C	negative	20

* A4T::pIG 121, C58::pIG 121 and LBA4404::pIG 121

Table 14 Results of co-cultivation experiments with *A. tumefaciens* and mature tissue of *C. annuum* L. ('Sweet banana')

Explant	Strains	Period of co-cultivation	GUS staining	Experiment number (Appendix G)
Mature plant leaf segment	A4T::pIG 121	48 hours in IM+C	negative	15
	C58::pIG 121		positive	
	LBA4404::pIG 121		positive	
Mature plant stem sections	A4T::pIG 121	48 hours in IM+C	negative	15
	C58::pIG 121		positive	
	LBA4404::pIG 121		positive	
Mature plants petals & anther	A4T::pIG 121	48 hours in IM+C	negative	15
	C58::pIG 121		positive	
	LBA4404::pIG 121		positive	
Stem and leaf from 2 week old plant	control	48 hours in IM+C	negative	17
	all*		negative	
Stem and leaf from 4 week old plant	control	48 hours in IM+C	negative	17
	all*		negative	
Stem and leaf from 8 week old plant	control	48 hours in IM+C	negative	17
	all*		negative	
Stem and leaf from 10 week old plant	control	48 hours in IM+C	negative	17
	all*		negative	

* A4T::pIG 121, C58::pIG 121 and LBA4404::pIG 121
* A4T::pIG 121, C58::pIG 121 and LBA4404::pIG 121
* A4T::pIG 121, C58::pIG 121 and LBA4404::pIG 121
* A4T::pIG 121, C58::pIG 121 and LBA4404::pIG 121

Plate 9.

Histochemical localisation of β-Glucuronidase in *C. annuum* L. ('Sweet banana') after co-cultivation with *A.tumefaciens* containing pIG 121

A. GUS activity in leaf (shown by arrow) after co-cultivation with C58::pIG 121

B. GUS activity in leaf after co-cultivation with LBA 4404::pIG 121

C. Cross-section of stem (control)

D. and **E.** GUS activity in stem (cross-section) after co-cultivation with C58::pIG 121

F. GUS activity in stem (longitudinal section) after co-cultivation with C58::pIG 121

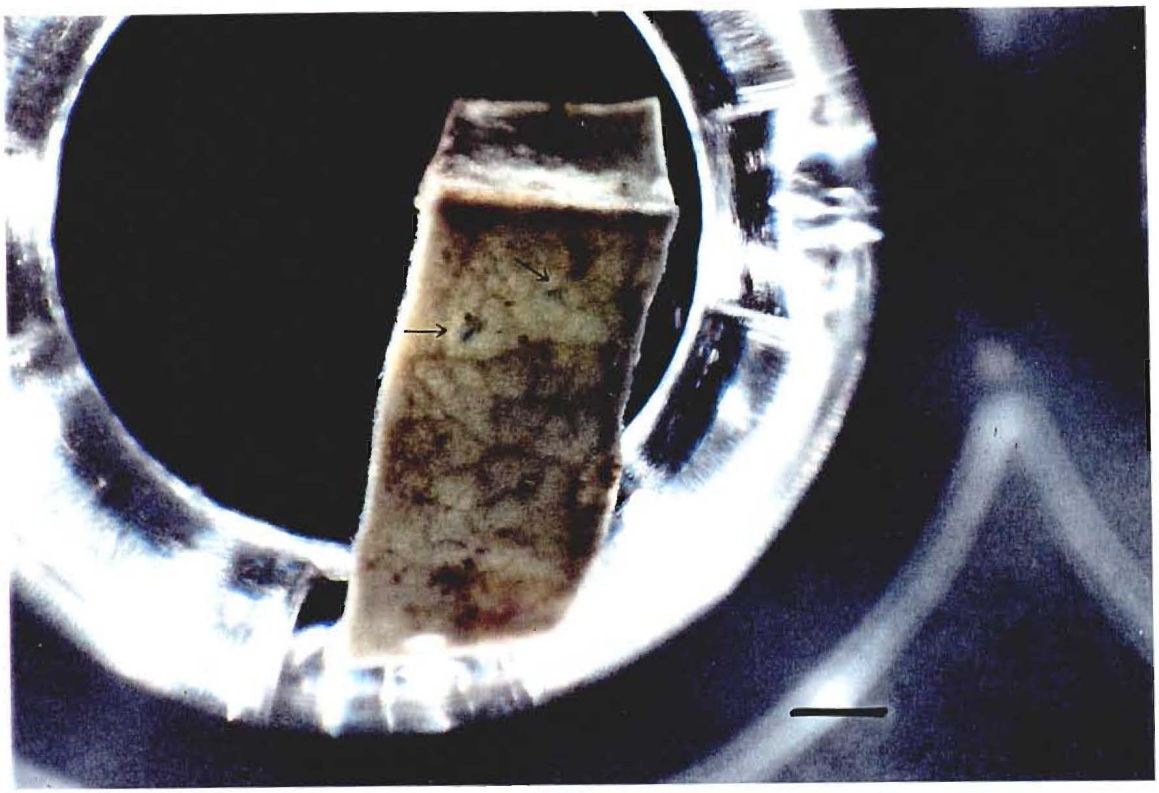
G. and **H.**
GUS activity in stem (cross-section) after co-cultivation with LBA4404::pIG121

I. Anther and petals (control)

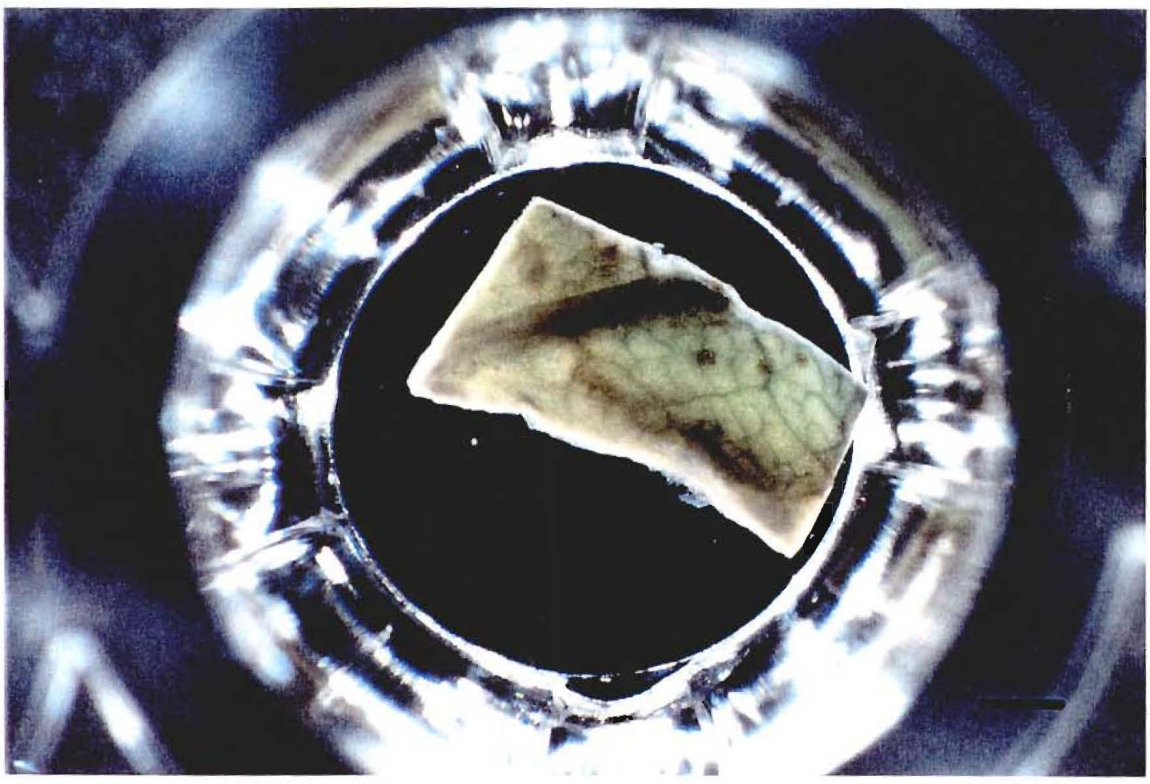
J. GUS expression in a complete anther after co-cultivation with C58::pIG 121

K. GUS-expressing petals after co-cultivation with C58::pIG 121

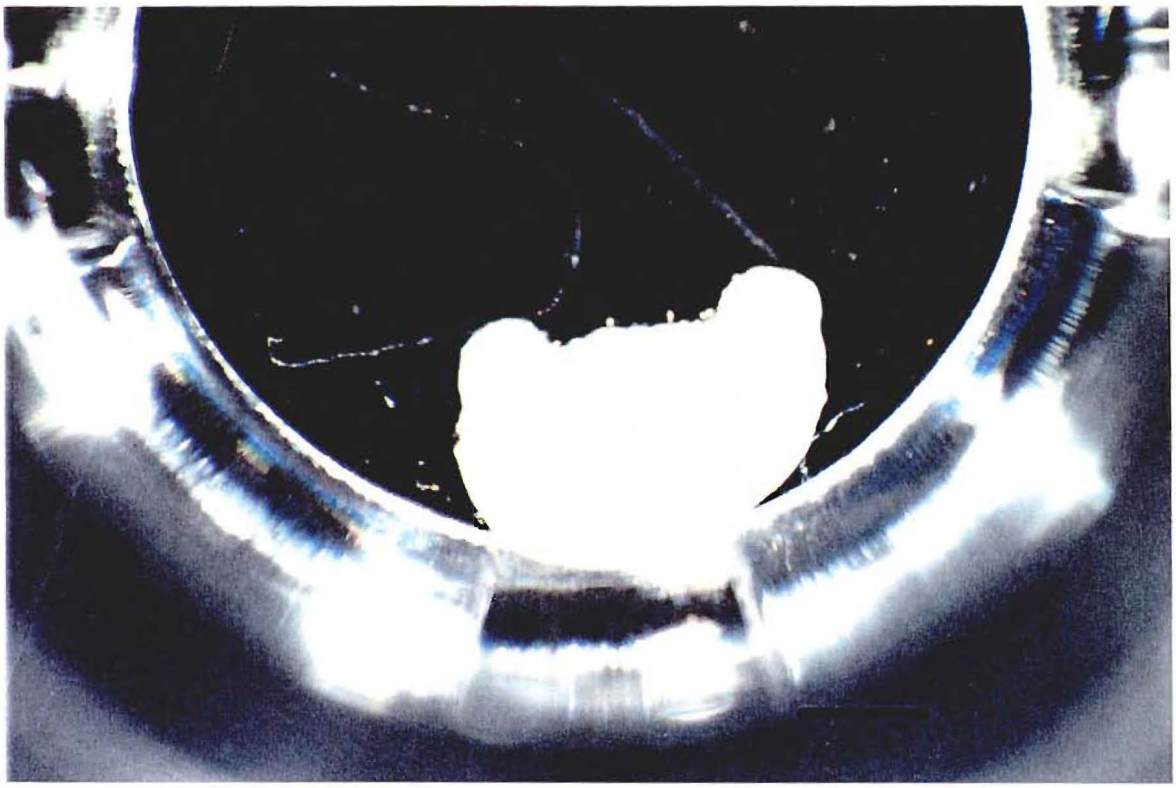
(bar = 1mm)



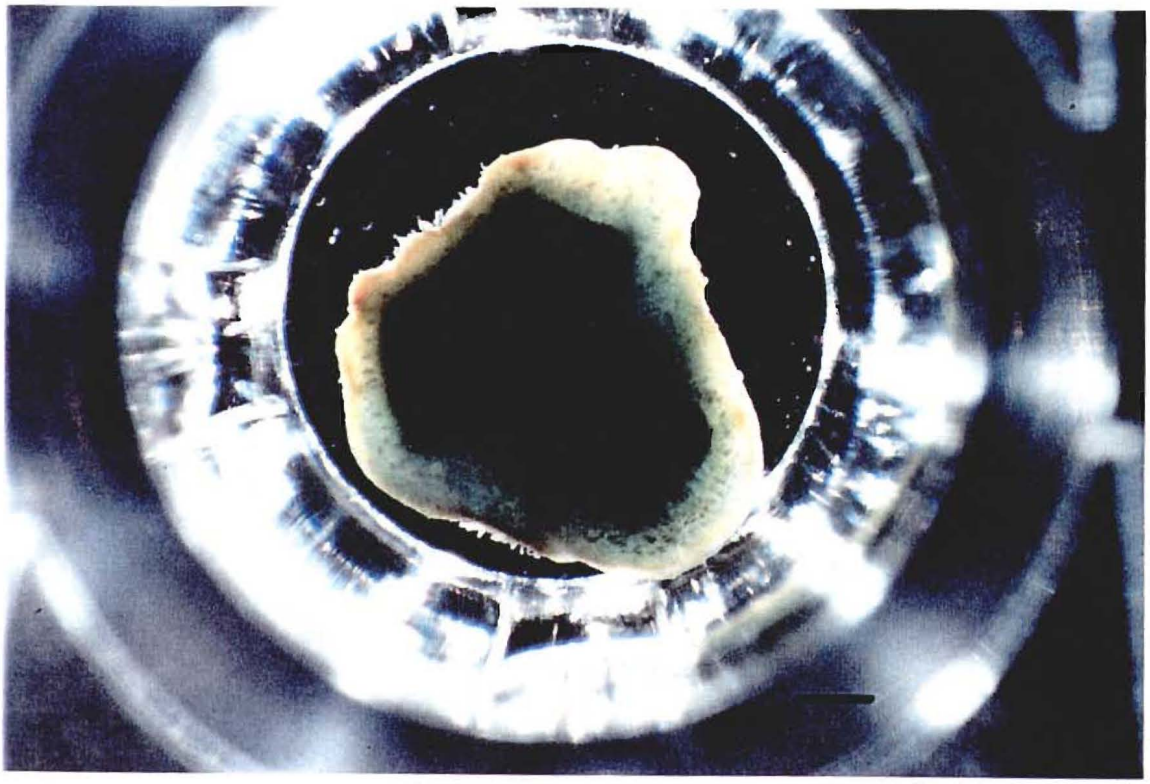
A



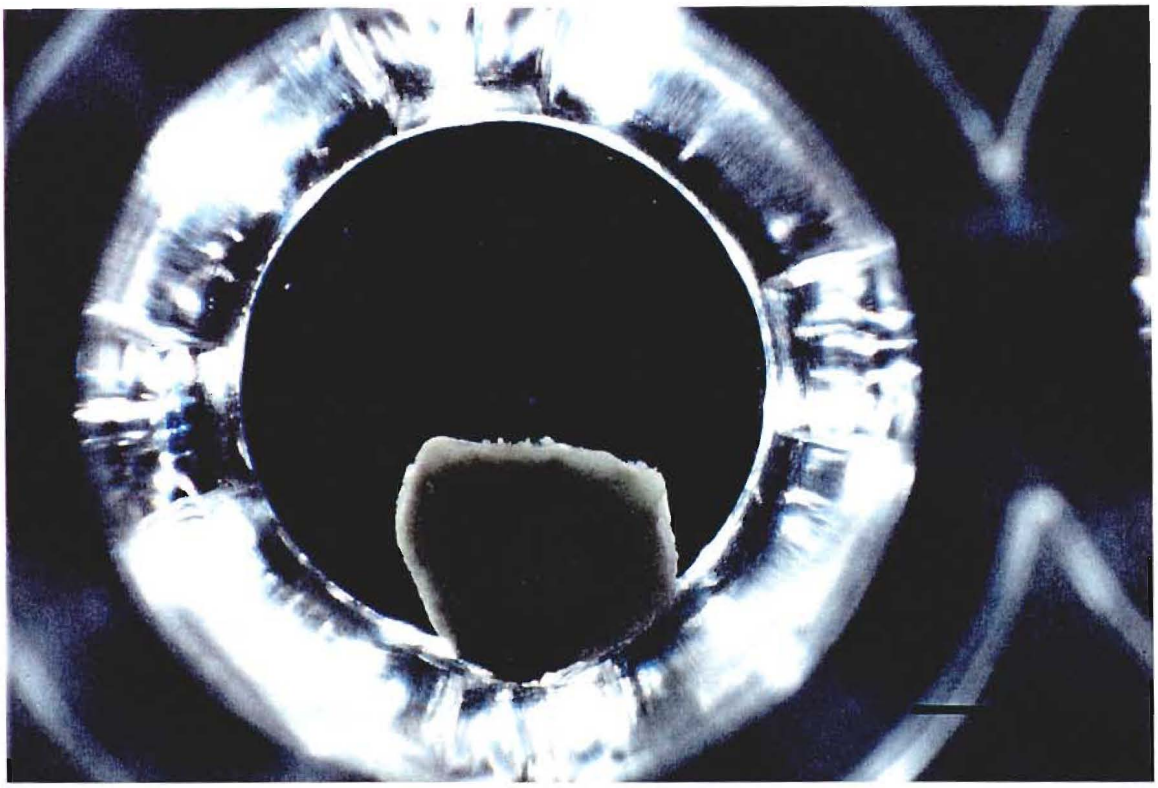
B



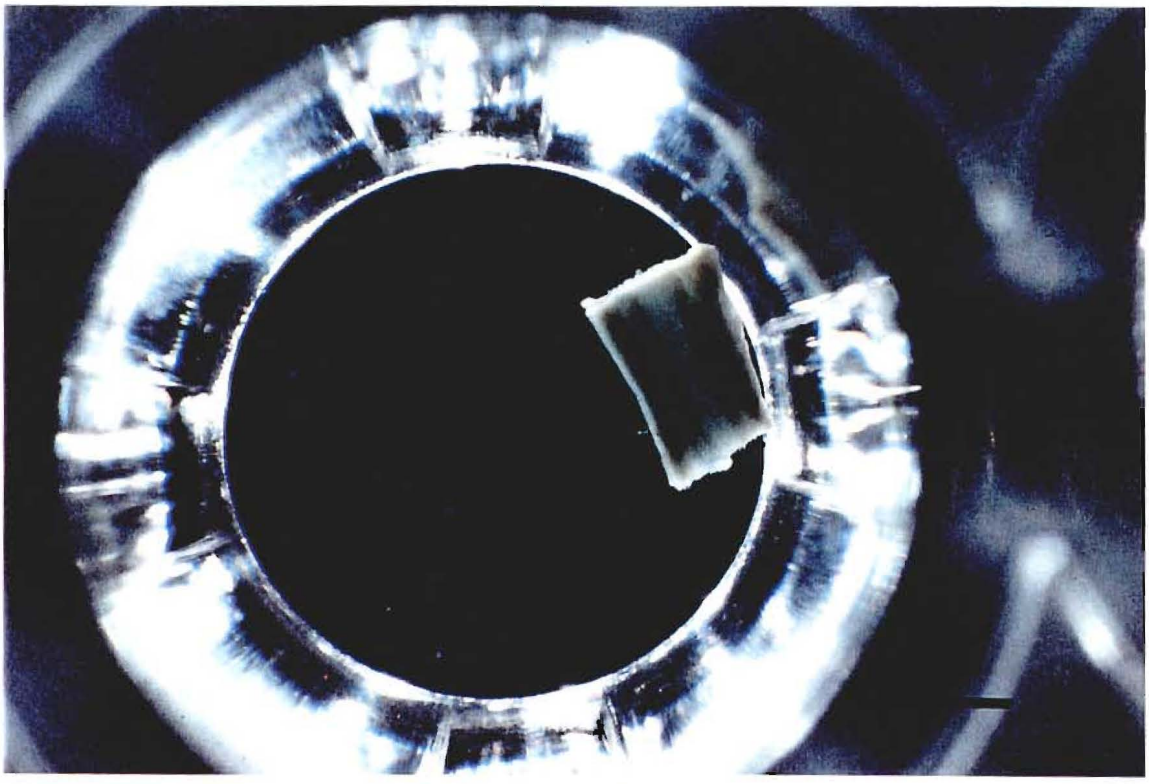
C



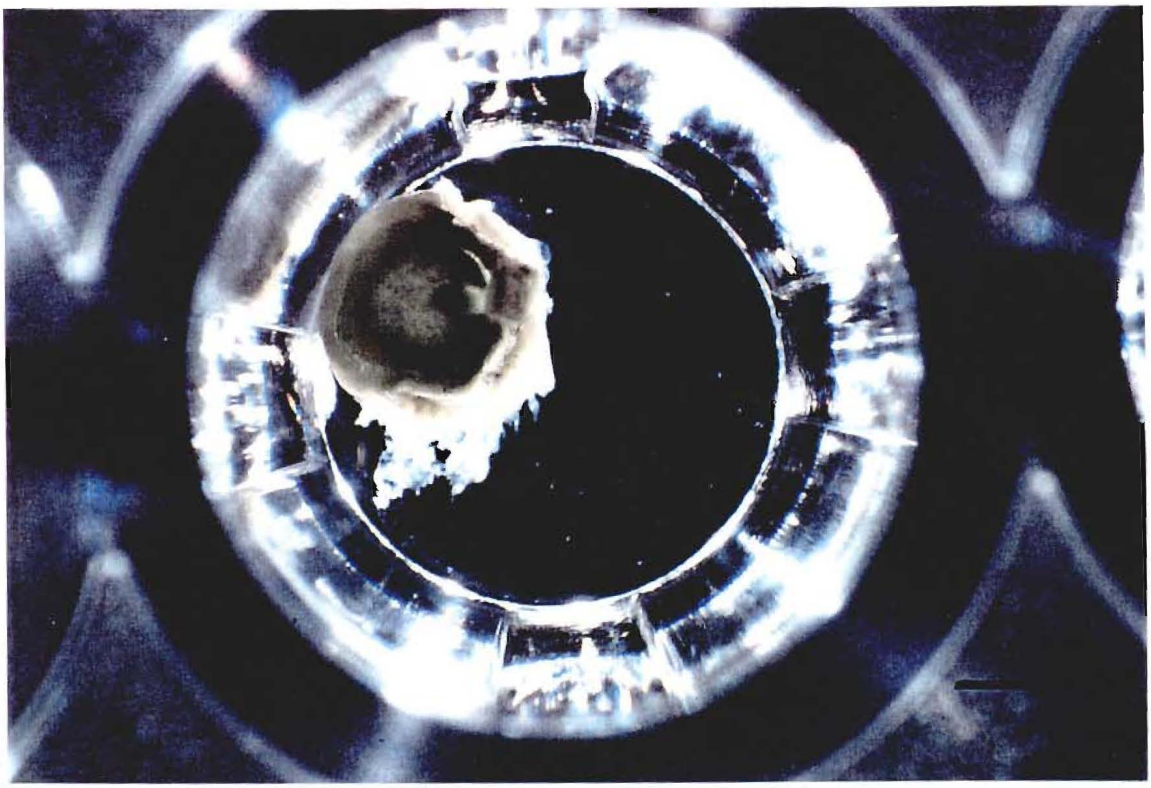
D



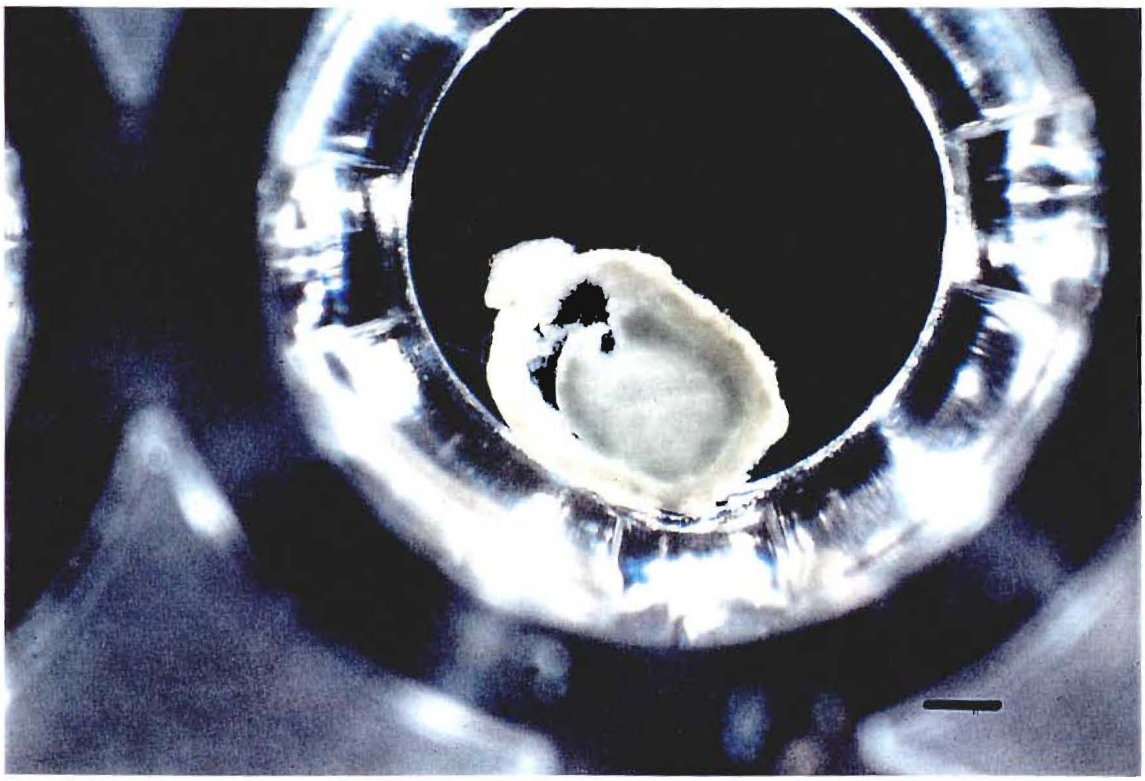
E



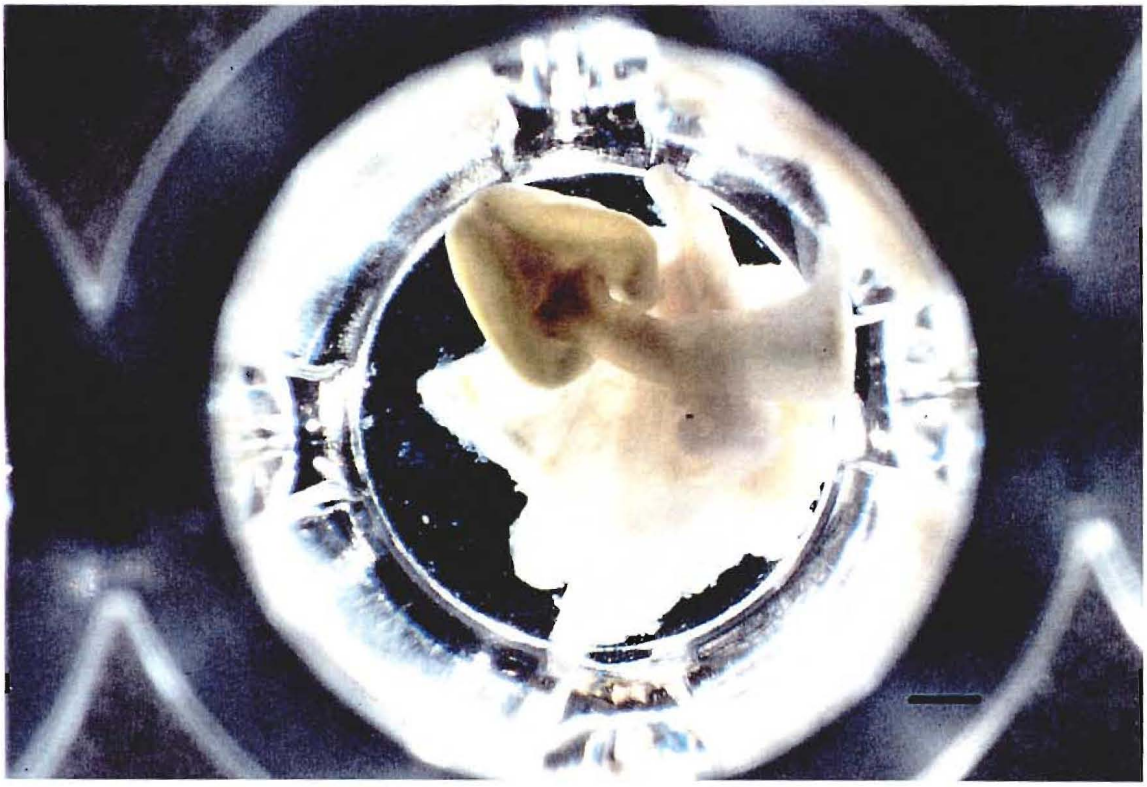
F



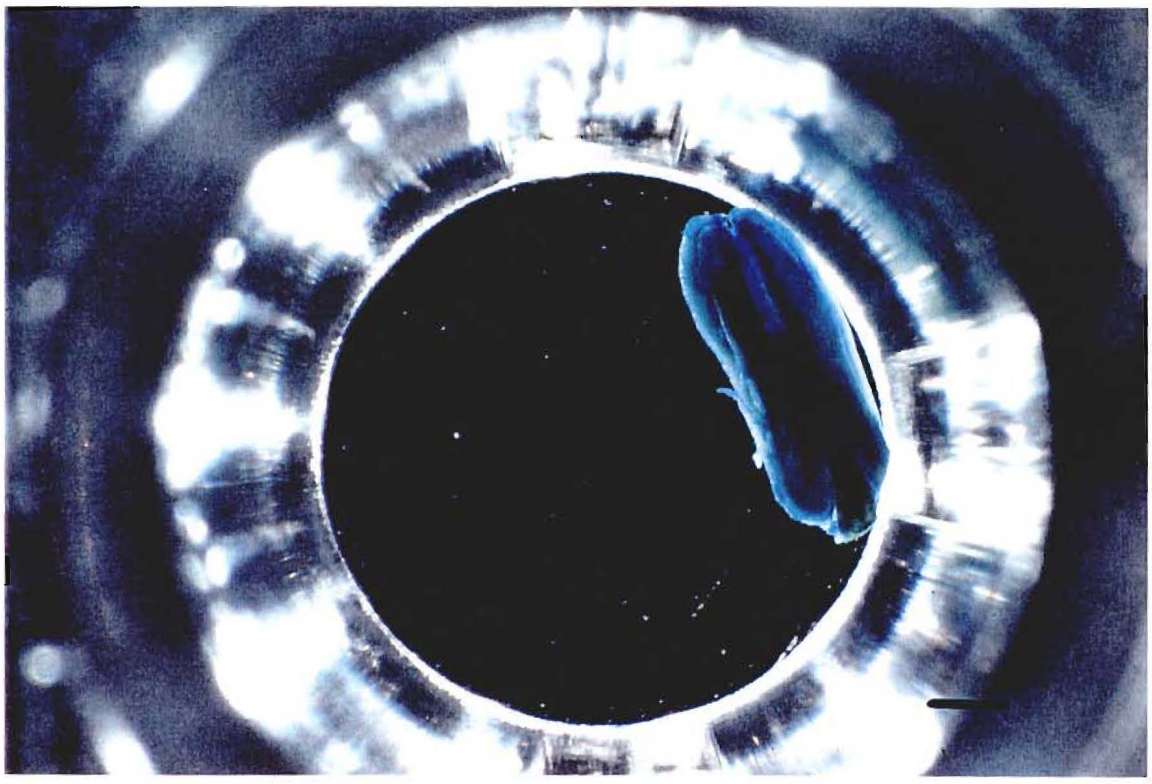
G



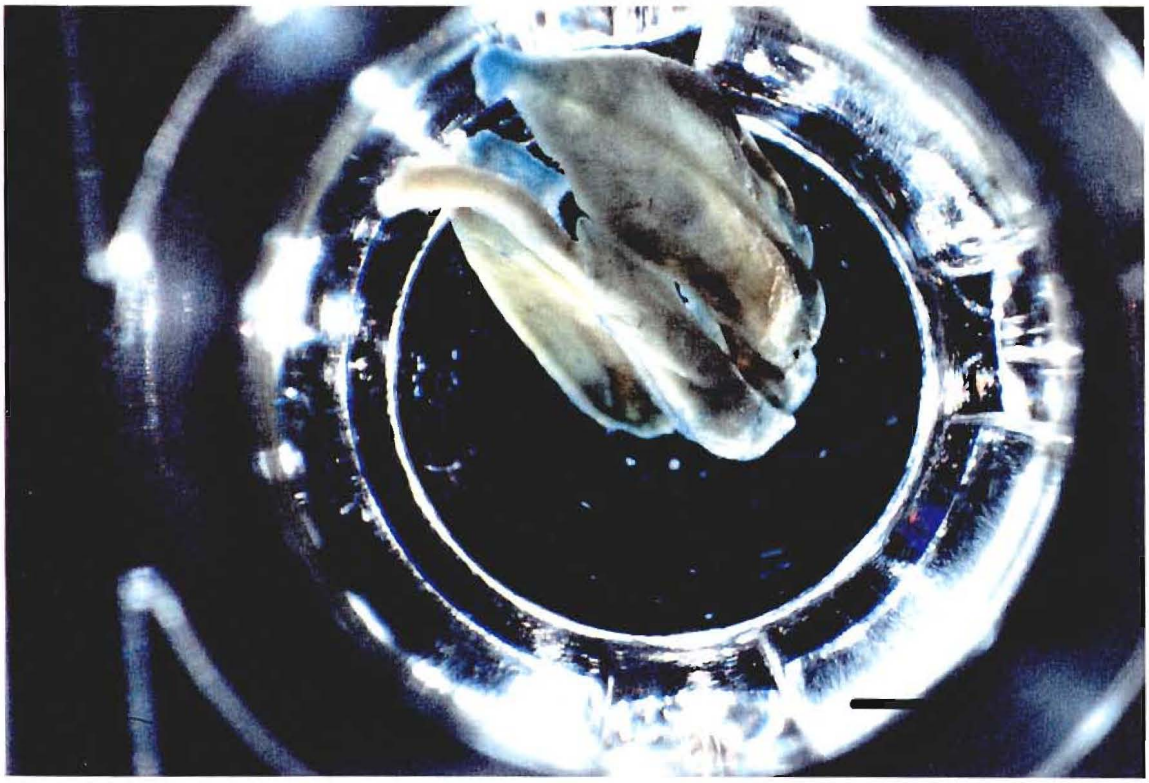
H



I



J



K

Plate 10.

Histochemical localisation of β-Glucuronidase in *Capsicum annuum* L. ('Yolo Wonder') after co-cultivation with *A. tumefaciens* containing pIG 121

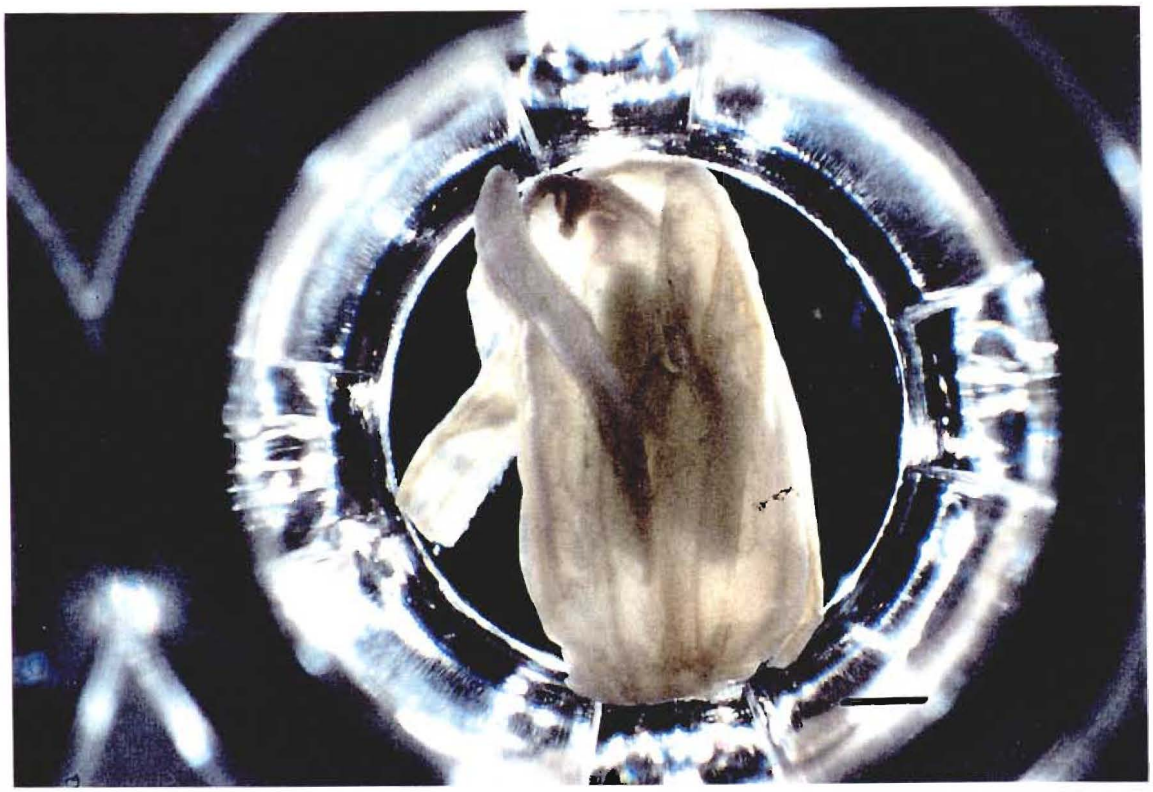
A. Control

B. Gus-expressing petals and anther (shown by arrow) after co-cultivation with C58::pIG 121

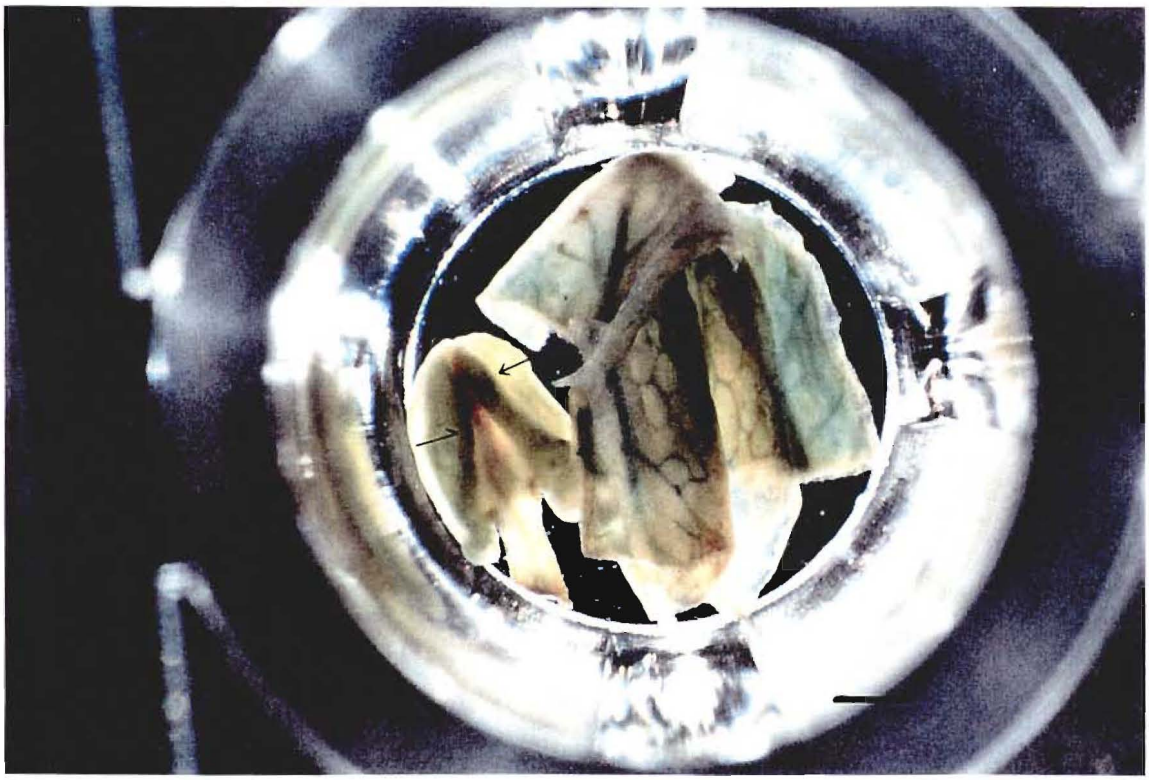
C. GUS-expressing petals after co-cultivation with LBA4404::pIG 121

D. GUS-expressing anther after co-cultivation with LBA4404::pIG 121

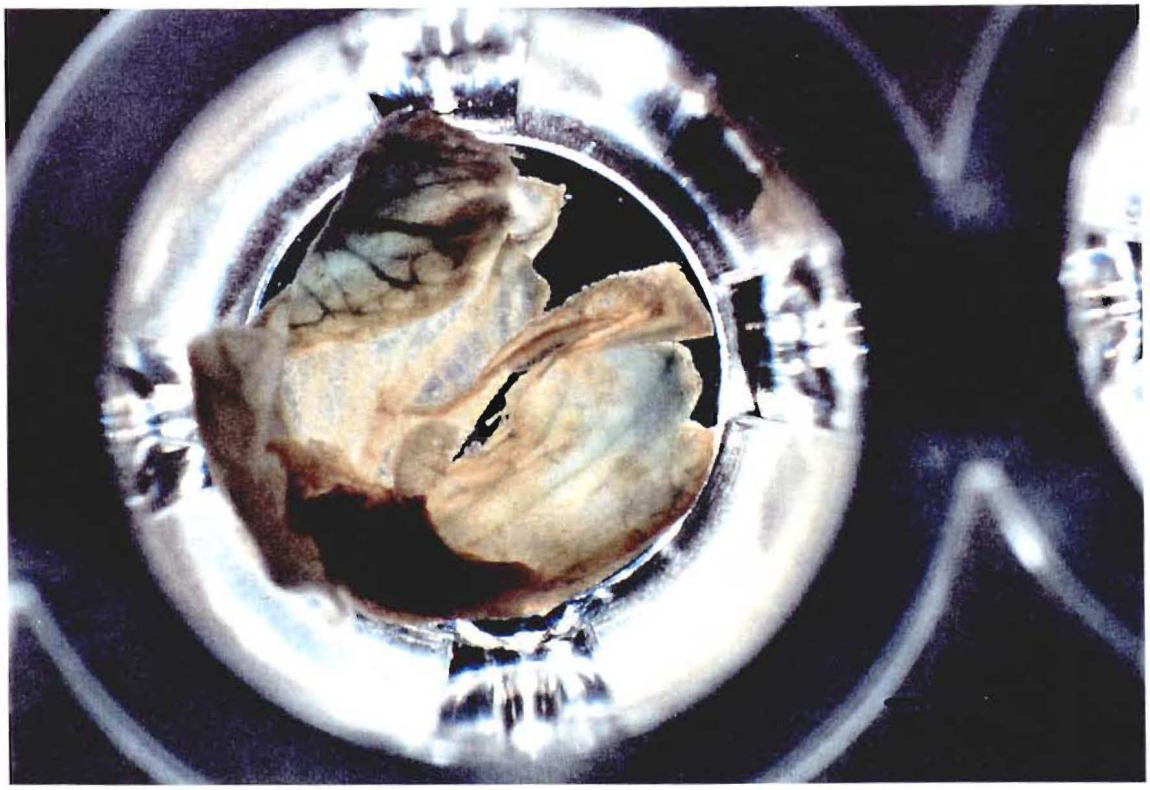
(bar = 2mm)



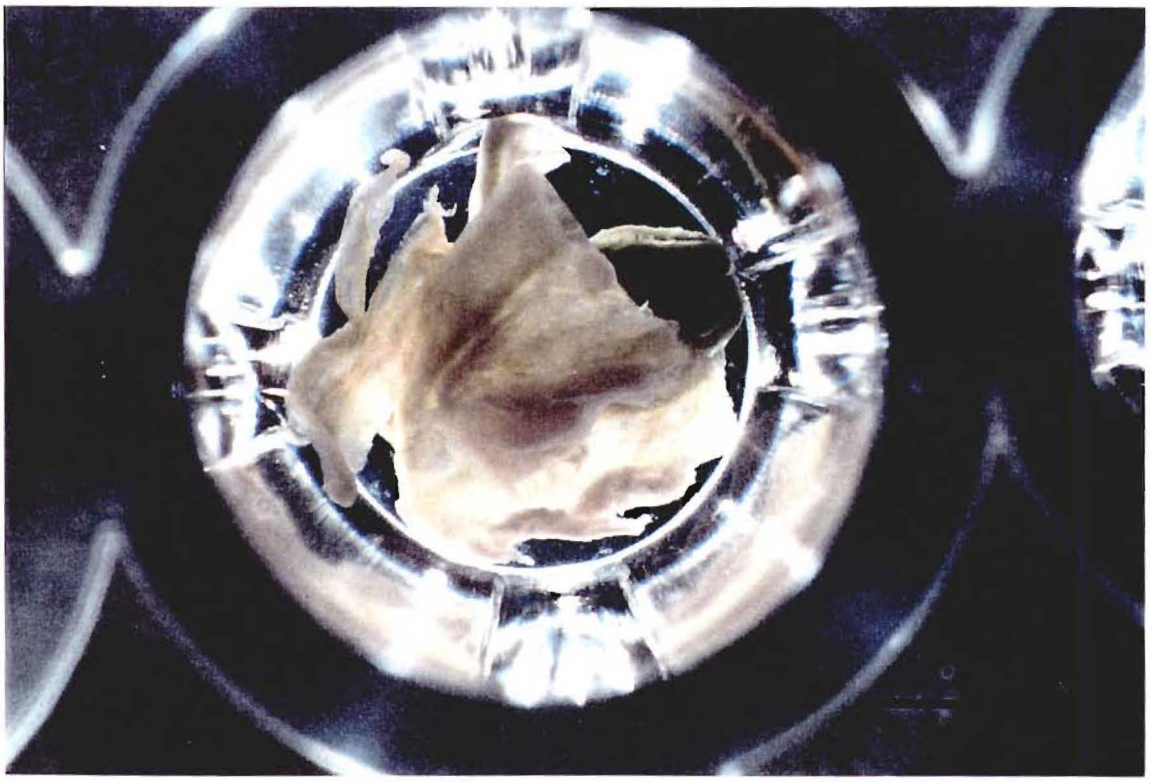
A



B



C



D

CHAPTER 4

DISCUSSION

4 DISCUSSION

The aim of the project was to develop an *Agrobacterium*-mediated transformation system for *Capsicum annuum* L. 'Sweet banana' and to define a regeneration procedure to establish transgenic plants. Figure 6 presents a schematic summary of the procedures used in this research towards achieving transgenic *C. annuum* L. ('Sweet banana')

For a number of *Capsicum* varieties regeneration of shoots from cotyledons and hypocotyl explants have been reported (Ebida and Hu, 1993; Arroya and Revilla, 1991). Ramage (1994) showed that in *C. annuum* L. ('Sweet banana') shoot induction occurs in the upper hypocotyl explant when it is cultured in medium containing 3%(w/v) sucrose and $5mgL^{-1}$ benzylaminopurine (BA). In preliminary experiments it was shown that when cultured in this shoot inductive medium (SIM) for 20 days, 80-100% of the upper hypocotyl explants formed a rosette of shoot buds. Ramage (1994) showed that a minimum of 8 days exposure to SIM is required to elicit a shoot forming response from the upper hypocotyl and the shoots were formed *de novo* from superficial layers of the explant without an intermediate callus phase.

The regeneration of shoots *de novo* from differentiating explant tissue without a callus phase means that the chances of somaclonal variation maybe reduced. The ability to transform this explant and induce transgenic shoot buds could lead to the production of transgenic plants. Any variation in phenotype of the trangenic cells and tissue would be due to the introduced genes.

To date, Liu *et al.* (1990) have been the only group to report transgenic tissue in a bell pepper variety of *C. annuum.* They obtained transformed shoot buds and leaf-like

structures that developed from a callus phase and expressed β-glucuronidase in the vascular and perivascular tissues. However they were unsuccessful in regenerating whole plants.

In peppers, regeneration from callus has been reported in only 2 instances, both involving regeneration from protoplasts (Saxena *et al.*, 1981 and Diaz *et al.*, 1988). The lack of a reliable system for regeneration of pepper plants *via* callus derived from non-meristematic explant tissues has hampered the development of an *Agrobacterium* based genetic transformation system such as the leaf-disc method used successfully for other Solanaceous genera (Horsch *et al.*, 1985 and McCormick *et al.*, 1986).

Another important consideration in the choice of this system for transformation work was that the developmental sequence leading to shoot formation was described by Ramage (1994) has now been confirmed. After 4 days in the inductive medium (SIM) the upper hypocotyl segment swells and elongates, loses its dark colouration and becomes light green. Between the 6th and 8th day of culture, the basipetal end of the upper hypocotyl was markedly more swollen than the acropetal end and wound callus is evident. By day 10 dark green 'spots' appear around the circumference of the cut ends. The green 'spots' are predominantly found on the basipetal end and these later become more visible as shoot primordia as they continue to develop further. At the end of 20 days a rosette of buds are formed (Plate 4).

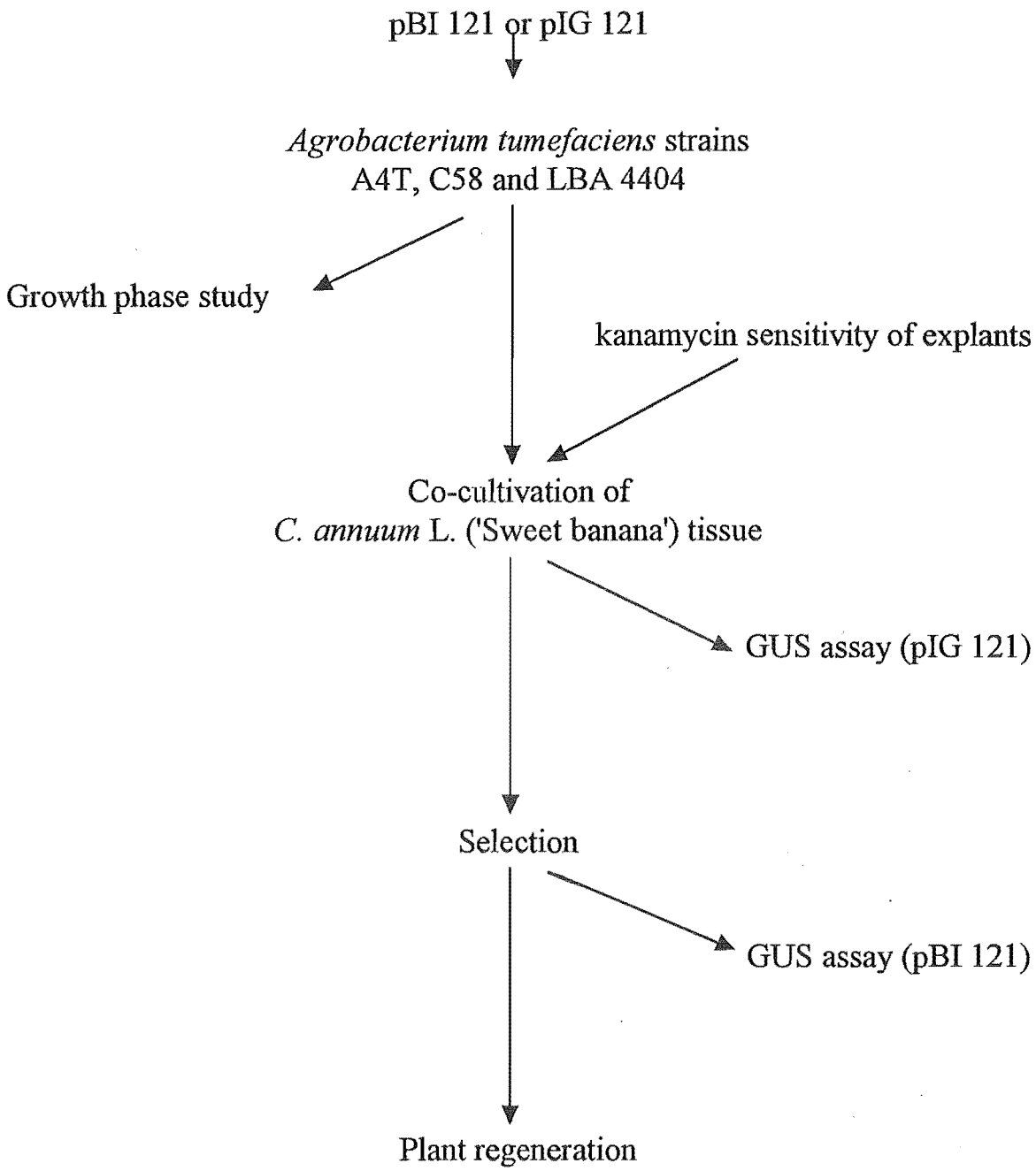


Figure 6 Schematic summary of procedural steps followed towards achieving *Agrobacterium*-mediated transformation of *C. annuum* L. ('Sweet banana')

Kanamycin sensitivity

Kanamycin resistance is the most widely used selectable marker for plant transformation (Colby and Meredith, 1990) and the sensitivity of a particular species or explant to kanamycin is an important element in the development of a new transformation system in which the kanamycin resistance gene will be used. Because sensitivity affects the recovery of transformed explants and varies widely among tissues (Colby and Meredith, 1990), it is important to test the concentration of kanamycin that can be used to recover transformants from untransformed explants.

The percentage of *C. annuum* L. ('Sweet banana') upper hypocotyl explants forming shoots was determined at different concentrations of kanamycin. Shoot induction and formation was inhibited by the antibiotic kanamycin when it is present in the medium in concentrations $50mgL^{-1}$ and higher. The upper hypocotyl elongates and swells but dark green 'spots' indicating the development of shoot bud formation was not observed. At kanamycin concentrations higher than $50mgL^{-1}$ the explant appeared bleached.

Kanamycin is an aminoglycoside antibiotic. Such antibiotics inhibit protein synthesis in prokaryotic cells by binding to the 30S ribosomal subunit thus inhibiting the initiation of translation and consequently, protein synthesis (Wilmink and Dons, 1993). Since ribosomes of mitochondria and chloroplasts of higher plants are related to bacterial ribosomes they are susceptible to kanamycin.

The fresh weight change in the explant was studied to determine whether there was a notable change in tissue development when compared with upper hypocotyl cultured inductive medium without kanamycin. At $50mgL^{-1}$ kanamycin change in fresh weight of the explant decreased drastically (Figure 2). The selection concentration of kanamycin was $50mgL^{-1}$ because it was the minimum concentration required to inhibit

shoot formation. Since the weight change is an increase over the original weight of the explants it indicated that the tissue was alive even at $200mgL^{-1}$, the highest concentration of kanamycin tested. Recent reports of using kanamycin to inhibit regeneration of untransformed explants in transformation work of other plant species include Yepes and Aldwinckle (1994) and Rakousky and Matousek (1994).

It was predicted that after the uptake of T-DNA, the kanamycin resistance marker gene would confer resistance to the cells of the explant enabling the upper hypocotyl to successfully undergo shoot formation in the presence of kanamycin whilst untransformed explants would not form shoot buds.

Transfer experiments showed that the inhibition of shoot induction at $50mgL^{-1}$ was stage specific. This was determined by transferring the explant from the shoot inhibiting medium (SIM+K) to the shoot inductive medium (SIM) daily. When the upper hypocotyl was cultured in SIM+K for the first 4 days, shoot induction was inhibited completely (Table 8). Less than 4 days culture in SIM+K allowed shoot formation to occur when it was transferred to SIM. This may have been due to a lack of new protein synthesis taking place in the explant so the protein inhibition by kanamycin was not evident. Synthesis of new proteins for the dedifferentiating and shoot inductive process in the explant may not be occurring until day 4.

Regeneration

As part of the preparatory work to producing transgenic plants, regeneration of the shoot buds into plantlets was studied. The shoot buds of the upper hypocotyl were subcultured further on appropriate media to induce roots and allow the leaf-like structures to develop. The buds were excised and cultured on MS media supplemented with

different combinations of NAA and IBA. However not all combinations were trialed owing to the outcome of the co-cultivation experiments and later due to time constraints.

The excised shoot buds retained the leaf-like structures without further development and formed roots when the MS medium was supplemented with 0.02 mgL^{-1} or 0.5mgL^{-1} IBA and 0.05mgL^{-1} NAA. The shoot buds excised from the upper hypocotyl and cultured on MS medium (without hormones) continued to develop leaf structures because of the carry over effect of the BA from SIM. Transferring these plantlets to root inducing medium may have resulted in root formation. Combinations of hormones tried resulted in extremes of root formation or callus induction. thus a likely sequence for obtaining plantlets after transformation would be to subculture the buds on MS medium and then transfer to root induction medium rather than directly transferring to medium supplemented with a combination of hormones (Plate 5).

Because of varietal differences, hormone combinations that suit one type do not necessarily have the same effect on another. Arroyo and Revilla (1991) regenerated plants from the rosette of shoot buds formed on hypocotyl from 4 week old seedling of a variety of bell peppers. The shoots were grown and rooted on medium containing 0.1mgL^{-1} NAA and 0.05mgL^{-1} IBA. Ebida and Hu (1993) had similar outcome with 'Early California Wonder' using younger explants and a hormone combination of 0.5mgL^{-1} IAA and 0.4 mgL^{-1} NAA.

The upper hypocotyl system

Advantages of the upper hypocotyl explant transformation and regeneration system are the ease in generatiing uniform sterile seedlings and in excising and co-cultivating large numbers of hypocotyl explant. 80-100% of the explant produces shoot

buds. Selection using this system appeared clearcut since untransformed upper hypocotyl explants would not produce shoot buds.

An important consideration when regenerating transgenic plants directly through shoot organogenesis is that this method has the potential to produce partially transformed plants. Such chimeras arise for example if *de novo* meristems form during co-cultivation with *Agrobacterium* and only one of several cells comprising the developing shoot is transformed (Voisey *et al.*, 1994). However chimeras are not likely to result in this system. The shoot buds arise directly from the superficial layers of the upper hypocotyl explant. A meristematic layer is formed initially and then adventitious shoot bud primordia develop from these cells. So when cultured in SIM+K+C only cells that are transformed and hence kanamycin-resistant, will be able to develop into shoot primordia. Shoot formation is inhibited in untransformed upper hypocotyl explants. So only the transgenic regenerated shoot buds can be excised and cultured on the appropriate medium to form whole plants. The regeneration of shoots directly from the explant tissue decreases the chances of somaclonal variation occurring (Voisey *et al.*, 1994). The upper hypocotyl therefore seemed to be a suitable target tissue for transformation.

Growth phases of *Agrobacterium*

A growth phase study of the 9 strains of *Agrobacterium* (Table 3) showed that between 12-20 hours after inoculation exponential growth occurred. The introduction of the binary plasmid did not affect the growth of the transformed strains. Differences in growth pattern were due to the difference in initial density of the inoculum used.

The *Agrobacterium* culture must be a rapidly growing one for transformations to be obtained (Burow *et al.*,1990). Clarke *et al.* (1992) showed that a rapidly growing culture is the most competent phase of the *Agrobacterium* for transformation. At higher

bacterial densities they obtained a two-fold increase in the number of transformed shoots produced. Also increasing the co-cultivation time from 48 hours to 72 hours improved the transformation frequencies at higher bacterial densities.

A balance is required between the availability of *Agrobacterium* cells sufficient to carry out T-DNA transfer and the problems associated with overgrowth of bacteria around the explant (Charest *et al.*, 1988 & McCormick *et al.*, 1986).

An overnight culture, growing for 12-16 hours, was used in all the experiments to ensure that the bacterial inoculum was in its rapid growth phase. Once a reliable transformation system of the upper hypocotyl explant was established then the optimum inoculation and co-cultivation periods, along with the most competent phase of the *Agrobacterium* could be determined to generate the highest frequency of transformation.

Binary vectors

Three *A. tumefaciens* strains (A4T, C58 & LBA 4404) transformed with the binary vector pBI 121 and pIG 121 (Table 3). The binary vector pBI 121 has within the T-DNA borders the 35S CaMV constitutive promoter, β-glucuronidase gene, and neomycin phosphotransferase (Figure 1). This vector can show positive GUS response if contaminating *Agrobacterium* are present in the vascular spaces or surrounding the explant.. When explants are tested positive for GUS it is imperative to check by microscopy to determine the location of the blue precipitation of the assay. pIG 121 contains an intron within the β-glucuronidase gene and the intron has a stop codon in the same reading frame as that of the enzyme. This prevents its expression in the *Agrobacterium* (Ohta *et al.*,1990).

Transformation of upper hypocotyl explants

C58 is the most virulent (Holford *et al.*, 1992) of the three original strains used and was found by Liu *et* al (1990) to be most effective for transformation of *C. annuum*. So this was chosen for the initial trials. The initial attempts at transformation of the upper hypocotyl using C58::pBI 121 were unsuccessful. The explants were inoculated for 60 minutes and blotted dry and placed in shoot inductive medium having the selective antibiotic and Claforan (SIM+K+C). Claforan was added to the medium to prevent the proliferation of the bacteria. At 200mgL^{-1} bacterial contamination was not evident and the explants were able to tolerate it (section 3.3). After 20 days the explants appeared slightly bruised at the cut ends suggesting that perhaps the bacteria had overcome the wounded plant cells during inoculation. For further experiments the period of inoculation was decreased to 30 minutes.

Co-cultivation with C58::pBI 121 and LBA4404::pBI 121 with the reduced time of inoculation also failed to produce shoots from the upper hypocotyl (Table 11). It was felt that the 30 minute exposure time for inoculation was insufficient and exposure to the culture for 60 minutes was too vigorous. So the explants were inoculated for 30 minutes and placed on initiation medium (solid SIM) to enable *Agrobacterium* that were associated with the explant to continue the interaction with the plant cells and thereby effectively increased the time frame required for T-DNA transfer process. To determine whether selection pressure at this stage affected the outcome, explants were incubated on medium with or without antibiotics (IM or IM+K+C). This co-cultivation procedure was carried out for 72 hours (after Clarke *et al.*, 1992) and then transferred to SIM+K+C.

The upper hypocotyls produced shoots and these appeared to be kanamycin resistant as they continued to develop in the selection medium. Since control explants had also formed shoots all these were suspected selection escapes. The shoots were resistant

to kanamycin ($50mgL^{-1}$) but were negative for GUS expression. The actual numbers of explants that were escapes are given in Appendix G but not evaluated further because the possible reason for the escapes was determined by 'reverse transfer' experiments and avoided in further trials.

The reverse transfer experiments (Table 9) showed that once the shoot induction pathway had been 'switched on' transfer to medium having kanamycin did not affect shoot formation. In this reverse transfer experiment upper hypocotyl explants were transferred daily from SIM to SIM+K whereas initially the transfer was from SIM+K to SIM. The percentage of explants forming shoots at the end of 20 days showed that when the explant was transferred after 3 days in SIM, 10% of the explants formed shoots. This trend continues for transfers until the 8th day. Transfer after day 8 results in 70% of explants forming shoots. The first 3-4 days are critical for the shoot induction process and kanamycin interferes at this stage. The complete inhibition of shoot induction by kanamycin occurrs around the 3rd or 4th day. But the upper hypocotyl once exposed to SIM for 8 days will continue to form shoots successfully. This minimum 8 day exposure to SIM for shoot formation supports the findings of Ramage (1994).

This ability of the shoots to continue regenerating in the presence of kanamycin after the induction has occurred explains how the escapes came about. The shoots once formed appear to be kanamycin-resistant at $50mgL^{-1}$. This shows that selection needs to be carried out from outset and explants need to be completely surrounded by the antibiotic. During the co-cultivation period of 72 hours the explants were resting on the surface of the solid medium hence the selection pressure was reduced as compared with the liquid SIM+K+C.

Cross-protection of non-transformed cells by transformed cells may also enable the shoot development to occur (Klee *et al.*, 1987). This possibility was unlikely in the light of the reverse transfer experiments. At this stage a GUS assay was sufficient to determine that the upper hypocotyls forming shoots were selection escapes even though the regenerated shoots were kanamycin-resistant. Southern hybridisation of plant DNA with the 3kbp GUS cassette could be carried out to confirm whether the uptake of T-DNA had occurred to render the shoots kanamycin-resistant.

When the co-cultivation period was decreased to 48 hours shoot formation did not occur. Varying the density of the inoculum had no effect either. Although it is highly regenerative, the upper hypocotyl explant of *C. annuum* L. ('Sweet banana') appears to be recalcitrant for *Agrobacterium*-mediated transformation.

GUS-Intron plasmid

At this juncture, the plasmid pIG 121 (Ohta *et al.*, 1990) was obtained. The advantages of this plasmid having an intron were discussed earlier. The expression of the marker gene can be detected within a few days of co-cultivation. This has the advantage of being able to monitor early events of transformation by studying the location of GUS expression (presence of blue spots) by light microscopy. It also reduces the time period required for the transformation experiments with the upper hypocotyl or other explants. Explants could be selected for competency without having first to develop a selection and regeneration protocols, a major improvement on the current stratergy for establishing transgenic plants. After extensive work on tissue culture techniques to regenerate plants from cell and organ tissue, before introduction of genes is avoided. Competent tissue can first be chosen then the regeneration of the explant can be developed.

There are few reports on the interaction of *Agrobacterium* with pepper tissues. De Cleene and De Ley (1976) noted only that *C. annuum* was susceptible to *Agrobacterium* strains B6 and chrIIB. More recently Liu *et al.* (1990) reported transgenic tissues but in a bell pepper variety. It can be noted from the literature sited (Chapter 1; section 1.4) that intervarietal differences in pepper result in explants responding differently to tissue culture. Since the upper hypocotyl of 'Sweet banana' seedlings was not susceptible to *Agrobacterium* infection it was imperative to determine whether the mature plant would be amenable to transformation.

Transformation of mature tissue

Leaf, stem and flower explants from 4 month old plants were inoculated for 60 minutes with 3 strains of *Agrobacterium* having the binary plasmid pIG 121 and then co-cultivated for 48 hours. The leaf and stem sections were amenable to tranformation with C58::pIG 121 and LBA4404::pIG 121 but not to A4T::pIG 121 indicating that *C. annuum* L. ('Sweet banana') may be strain specific. The petals and anthers expressed GUS only when co-cultivated with C58::pIG 121 (Plate 9). *C. annuum* L.('Yolo Wonder') also proved to be susceptible to the *A. tumefaciens* strains. Only the petal and anther tissue was tested (Plate 10).

Several attempts were made to repeat the successful tranformation of mature explants and vary some of the factors to optimise transformation. But GUS expression was obtained on only 2 occassions with leaf and stem explants. Flower explants were more readily transformable; GUS expression was obtained three times. A variables leading to negative results could be the loss of the plasmid carrying the marker gene. This could occur if the growth room temperature was to rise above 30°C (Chen *et al.*, 1994) as had been subsequently discovered.

Pre-conditioning of explants

Studies have shown that competence was related to a brief duration of cell division at the wound site (An *et al.*, 1985). Transformation occured during this critical phase of cell division but remains ill defined. This is mainly because competent cells could not be identified precisely without the use of a detectable marker or reporter gene. Several reporter genes exist but the β-glucuronidase gene is an excellent marker as most plants lack endogenous activity (Jefferson *et al.*, 1987) and with plasmid pIG 121, competent cells within the upper hypocotyl could be studied.

Having established that the 'Sweet banana' variety was amenable to *Agrobacterium*-mediated transformation it was decided to target the upper hypocotyl explant again for transformation. The upper hypocotyl was inoculated for 60 minutes and then co-cultivated for 48 hours with *A. tumefaciens* containing pIG 121 (GUS-intron) and still the GUS assay was negative (Experiment 21; Table 12). Since this explant was proving to be difficult to transform the upper hypocotyl explant was first cultured in SIM for a defined period of time before inoculation with *Agrobacterium*.

This pre-conditioning treatment could improve the ability of the upper hypocotyl explant to take up T-DNA. Chyi and Phillips (1987) increased putatively competent cells by a pre-culturing treatment indicating that cell activation and division following wounding were insufficient for transformation without phytohormone treatment. Sangwan *et al.* (1991) observed that abundant transformation required not only cell activation (an early state exhibiting an increase in nuclear protein) but also cell proliferation and non-competent cells could be made competent with the appropriate phytohormone treatments before bacterial infection. Studies by Gheysen *et al.* (1987) on the integration of the T-DNA into the plant nuclear genome strongly suggest that host DNA synthesis is

required. Thus the ability of the host cell to enter one or more cell cycles may be required for successful transformation.

Pre-conditioning the upper hypocotyl explant (for 4 days or 8 days in SIM and 4 days in SIM+K) and then challenging with *Agrobacterium* did not produce transformants. Pre-conditioning did not change the competency state of the upper hypocotyl cells. This reinforces the initial findings that while the upper hypocotyl of the 11-day old seedling is an ideal explant for regeneration of shoots *de novo* it is recalcitrant for *Agrobacterium*-mediated transformation.

Successful *Agrobacterium*-mediated transformation and regeneration systems have been reported for a variety of plants. Leaf-discs from petunia, tobacco and tomato are as receptive to *Agrobacterium* (Horsch *et al.*,1985) as the hypocotyl of 5 day old seedling of *Brassica napus* L. (Radke *et al.*, 1988) or *Arabidopsis* explants (Sangwan *et al.*, 1991). The only plant tissue attribute common to the systems is that there is a callus phase from which regeneration of shoots occurs. Reports of tissue culture in *Capsicum* show that although callus growth was common at the cut surfaces shoot buds regenerated directly from the explant tissues without the intervention of callus growth (Agrawal *et al.*, 1989). Unorganized calli from pepper seedling explants are not capable of *de novo* shoot bud regeneration (Ebida and Hu, 1993; Agrawal *et al.*, 1989 and Phillips and Hubstenberger, 1985). Voisey *et al.*(1994) produced transformed shoots directly from hypocotyl explants at a low frequency (less than 1%) in *Trifolium repens* L., commonly called white clover. They observed that partial transformation had occurred in some of the plants produced.

Factors that affect transformation

While there is increased understanding of the interaction between the T-DNA of *A. tumefaciens* and the plant genome at a molecular level (Zambryski, 1992), very little is known at the cellular level (Binns, 1990).

Crucial factors for transformation include: (i) recognition and attachment of virulent bacterial cells to specific sites of the plant cell surface (Neff and Binns, 1985); (ii) expression of *vir* genes of the Ti plasmid that control the transfer of the T-DNA region, induced by substances secreted by susceptible plant cells (Stachel *et al.*, 1985) and (iii) plant cell competence for transformation which requires a high rate of plant DNA synthesis and cell division (An *et al.*, 1985).

Polysaccharide threads *A. tumefaciens* attach to the outer portion of the competent host cell wall (a pre-requisite to any subsquent transfer of Ti plasmid from the bacteria to plant cell). Each competent cell contains binding sites recognizable by *A. tumefaciens*. If this polysaccharide binding site contact is blocked then transformation is blocked (Sangwan *et al.*, 1991). The cuticle inhibits contact so epidermal cells do not undergo transformation.

Ramage (1994) showed that shoot regeneration of upper hypocotyl explant in 'Sweet banana' was directly from the superficial layer without intervening callus phase. Hence this explant may not be amenable to *Agrobacterium* interaction. Pre-culture should however have exposed dividing cells to the bacteria.

Plant transformation requires a wound response by the host cell. Broadly speaking most dicot plants respond to wounding by inducing adventitious cell proliferation with the

formation of a 'wound periderm' or a boundary layer and by producing phenolic molecules such as acetosyringone and hydroxyacetosyringone.

These phenolic molecules induce *vir* genes, a clearly defined step in transformation (Zambryski, 1992 and Stachel *et al.*, 1985). Plant metabolites capable of inducing the virulence genes of *Agrobacterium* can be determined by a thin layer chromatography technique developed by Sahi *et al.* (1994). This technique can be used to compare the virulence gene inducers of different plant species as well as extracts from different explants of the same species. It would help explain variation in competency of different plant tissue types and determine which tissue types should be targeted for transformation. The method of detection uses an *A. tumefaciens* strain containing a plasmid with an inducible virulence gene fused to a galactosidase gene (*vir*E::*lacZ*). Thin layer chromatography plates were overlayed with agar containing the indicator bacterium and a chromagenic galactoside. Virulence gene inducing metabolites induce the galactosidase and show a blue pigment change (Sahi *et al.*, 1994). In the case of *C. annuum* L. ('Sweet banana') the upper hypocotyl cellular metabolites could have been compared to the meatabolites of the mature plant tissue. If inducing metabolites were present in the mature tissue and not in the upper hypocotyl then such inducers could be added to the co-cultivation medium. Holford *et al.* (1992) demonstrated that by optimising a wide range of culture conditions such as altering the pH and adding phenolic compounds when working with new genotypes, *Agrobacterium* -mediated transformation systems can be developed for new species or varieties.

Sangwan *et al.* (1991) found that dedifferentiation is required for transformation of cotyledons, leaves and roots of *Arabidopsis*. In several other plant species regenerated directly from totipotent epidermal or subepidermal layers (without undergoing dedifferentiation) of *Agrobacterium* infected cotyledon or hypocotyl explants are not

transformed (Sangwan *et al.*, 1991). In *Linum*, Basiran *et al.* (1987) recovered transformed shoots from green callus that had proliferated at the cut surface and had gone through dedifferentiation prior to transformation.

Thus a likely sequence of events leading to the production of transformed cells and shoots would appear to be that cells pass through a period of dedifferentiation during which time they are permissive to transformation by *Agrobacterium*. Sangwan *et al.* (1991) suggest that a pre-culture treatment should be applied as a rejuvenating treatment to create or enhance transformation efficiency in recalcitrant or difficult-to-transform plant species. Given the correct phytohormone treatment cells have the potential to shift to the competent state.

Effect of development stage

Co-cultivation experiments were carried out with explants from 2, 4, 8 and 10 week old plants to bridge the gap between the upper hypocotyl explant of 11-day seedling and the mature 4 month old plant. This was carried out to determine if the competency of the tissue to be transformed was acquired as the plants matured. The negative GUS results suggest that juvenile tissues were not competent for transformation until much older.

Many researchers have highlighted the importance of type and age of tissue on the efficiency of gene transfer (De Kathen and Jacobsen, 1990; James *et al.*, 1990 and Armstead *et al.*, 1987). Meristematic tissue with actively dividing cells is found in most cases to be the most susceptible starting material. Seedling explants or embryos have often been used effectively with both oncogenic and disarmed strains (van Wordragen and Dons, 1992). Leaves from young plantlets are a better source of explants than older leaves (Schmidt and Willmitzer, 1988). Contrary to this Pang and Sanford (1988)

reported efficiently transforming older leaves of papaya than younger leaves or cotyledons. Davis *et al.* (1991) found that in tomato plants older leaves and cotyledons were slightly more susceptible than younger leaves.In both cases oncogenic strains were used.

Since the mature tissue of *C. annuum* L. ('Sweet banana') was amenable to transformation, a regeneration protocol for leaf and stem explants needs to be developed. Regenerating haploid plants from anthers of pepper plants have been reported (Dumas de Vaulx and Chambonnet, 1979 in Reynolds, 1986).

Biology of host limitation to *Agrobacterium*-mediated transformation

Agrobacterium based strategies for transformation are in widespread use because of the efficiency with which transformation occurs and the simplicity of the plant transformation and selection system. This system is highly host specific. The characteristic feature of the most widely used host plants is that a well developed tissue culture and regeneration methodology is available and that plant cells competent to regenerate are also competent to be transformed. The ability to transform tissue explants followed by immediate selection and regeneration has provided a standard method in the utilization of transgenic plants to study gene regulation (Horsch *et al.*, 1985) in model plant systems, namely tobacco, petunia and *Arabodopsis.*

While this system has been of some use in the study of plant gene expression it is clear that some hosts are not at all transformed by this system. The natural host range of *Agrobacterium*-originally defined by its ability to initiate tumours on plants (DeCleene and DeLey, 1976) - does not include most monocotyledons and is severly attenuated on many agronomically important dicotyledons such as soybean (Binns, 1990). Also some varieties of the same species can have vastly different capacities to be transformed (Binns, 1990).

A major group of agronomically important plants, the cereal crops are recalcitrant to *Agrobacterium*-mediated transfromation. The reason for this may be the wound response typical of monocotyledons. In most other plants when wounded, the cells adjacent to the wound site dedifferentiate and can in the process incorporate foreign DNA and then regenerate callus. In cereals wounding leads to the death of cells adjacent to the wound site. This limitation in recalcitrant monocotyledons and other plant types can be overcome by transformation techniques that do not depend on a wound response. For example wheat cultivars have been transformed using 'biolistics' ; the technique of bombarding plant tissue or cells with DNA-coated particles at high velocity (Vasil *et al.*, 1992). Other methods are direct gene transfer, in which naked DNA is introduced into protoplasts using polyethylene glycol or electroporation and micro-injection, in which naked DNA is inserted mechanically into protoplasts or intact cells (Potrykus, 1991). Since protocols for regeneration of pepper plants from protoplasts have been reported previously by Saxena *et al.* (1981) and Diaz *et al.* (1988) these alternative techniques for transformation could be developed.

In conclusion, *C. annuum* L. ('Sweet banana') was amenable to *Agrobacterium*-mediated transformation and has been shown here to express introduced genes. Transformation was particularly successful with explants from mature tissue. The explant originally targeted for transformation was the upper hypocotyl of an 11-day old seedling. This explant proved recalcitrant to *Agrobacterium*-mediated transformation. Further research could be carried out to develop a protocol for leaf-disc transformation and regeneration in *C. annuum* using the transformable mature tissue. Alternatively, other transformation techniques could be developed to transform the upper hypocotyl explant as it can be easily regenerated.

REFERENCES

Agrawal S., Chandra N. and Kothari S.L. (1989). Plant regeneration in tissue cultures of pepper (*Capsicum annuum* L.cv.'Mathania'). *Plant Cell Tissue and Organ Culture* **16:** 47-55

Agrawal S., Chandra N. and Kothari S.L. (1988). Shoot tip culture of pepper for micropropagation. *Current Science* 57(24): 1347-1349

Agrawal S. and Chandra N. (1983). Differentiation of multiple shoot buds and plantlets in cultured embryos of *Capsicum annuum* L.var.'Mathania' . *Current Science* **52**(13): 645-646

An G., Watson B.D., Stachel S., Gordan M.P. and Nester E.W. (1985). New cloning vehicles for transformation of higher plants. *EMBO J.* 4(2): 277-284

Andrews J. (1984). *Peppers; the domesticated* Capsicums. University of Texas Press, Austin. 170p.

Armstead I.P. and Webb K.J. (1987). Effect of age and type of tissue on genetic transformation of *Lotus corniculatus* by *Agrobacterium tumefaciens. Plant Cell Tissue and Organ Culture* **9:** 95-100

Arroyo R. and Revilla M.A. (1991). *In vitro* plant regeneration from cotyledon and hypocotyl segments in two bell pepper cultivars. *Plant Cell Reports* **10**: 414-416

Balestrazzi A., Carbonera D. and Cella R. (1991). Transformation of *Daucus carota* hypocotyl mediated by *Agrobacterium tumefaciens* . *Journal of Genetics and Breeding* **45**: 135-140

Basiran N., Armitage P., Scott R.J and Draper J. (1987). Genetic transformation of flax (*Linum usitatissimum*) by *Agrobacterium tumefaciens*: Regeneration of transformed shoots via a callus phase. *Plant Cell Reports* **6**: 396-399

Bercetche J., Chriqui D., Adam S. and David C. (1987). Morphogenetic and cellular reorientations induced by *Agrobacterium rhizogenes* (strains 1855, 2659 and 8196) on carrot, pea and tobacco. *Plant Science* **52**: 195-210

Bevan M. (1984). Binary *Agrobacterium* vectors for plant transformation . *Nucleic Acids Research* **12** (22): 8711-8721

Binns AN. 1990. *Agrobacterium* - mediated gene delivery and the biology of the host range limitations. *Physiologia Plantarum* **79**: 135-139

Binns, AN. and Thomashow, M. 1988. Cell biology of *Agrobacterium* infection and transformation of plants. *Annual Review of Microbiology* **42**: 575-606

Burow M.D., Chlan C.A., Sen P., Lisca A. and Murai N. (1990). High-frequency generation of transgenic tobacco plants after modified leaf disk co-cultivation with *Agrobacterium tumefaciens* . *Plant Molecular Biology Reporter* **8** (2): 124-139

Caplan A., Herrerra-Esrrella L., Inzé D., van Haute E., van Montagu M., Schell J. and Zambryski P. (1983). Introduction of genetic material into plant cells. *Science* **222**: 815-821

Charest P.J., Holbrook L.A., Gabard J., Iyer V.N. and Miki B.L. (1988). *Agrobacterium*-mediated transformation of thin cell layer explants from *Brassica napus. Theor. Appl. Genet.* **75**: 438-445

Chee P.P. (1990). Transformation of *Cucumis sativus* tissue by *Agrobacterium tumefaciens* and the regeneration of transformed plants. *Plant Cell Reports* **9**: 245-248

Chen H., Nelson R.S. and Sherwood J.L. (1994). Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze thaw transformation and drug selection. *Biotechniques* **16** (4): 664-668

Chilton M-D., Saiki R.K., Yadav N., Gordan M.P. and Quétier F. (1980). T-DNA from *Agrobacterium* Ti plasmid is in the nuclear DNA fraction of crown gall tumour cells. *Proc. Natl. Acad. Sci.(USA)* **77**: 4060-4064

Christopher T. and Rajam M.V. (1994). *In vitro* clonal propagation of *Capsicum* spp. *Plant Cell Tissue and Organ Culture* **38**: 25-29

Christopher T., Prolaram B., Rajam M.V. and Subhash K. (1986). Plantlet formation in embryo cultures of *Capsicum annuum* L.var.G4. *Current Science* **55**(20): 1036- 1037

Chyi Y-S. and Phillips G.C. (1987). High efficiency *Agrobacterium* -mediated transformation of *Lycopersicon* based on conditions favourable for regeneration. *Plant Cell Reports* **6**: 105-108

Clarke M.C., Wei W. and Lindsey K. (1992). High frequency transformation of *Arabidopsis thaliana* by *Agrobacterium tumefaciens* . *Plant Molecular Biology Reporter* **10** (2): 178-189

Colby S.M. and Meredith C.P. (1990). Kanamycin sensitivity of cultured tissues of *Vitis*. *Plant Cell Reports* **9**:237-240

Conner A.J., Williams M.K., Gardener R.C., Deroles S.C. Shaw M.L. and Lancaster J.E. (1991). *Agrobacterium*-mediated transformation of New Zealand potato cultivars. *New Zealand Journal of Crop and Horticultural Science* **19**: 1-8

Davis M.E., Lineberger R.D. and Miller A.R. (1991). Effect of tomato cultivar, leaf age and bacterial strain on transformation by *Agrobacterium tumefaciens*. *Plant Cell Tissue and Organ Culture* **24**: 115-121

De Cleene M. and De Ley J. (1976). The host range of crown gall. *Botanical Review* **42**: 389-466

De Kathen A. and Jacobsen H.J. (1990). *Agrobacterium tumefaciens*-mediated transformation of *Pisum sativum* L. using binary and cointegrate vectors. *Plant Cell Reports* **9**: 276-279

De Paolis A., Mauro M.L., Pomponi M., Cardarelli M., Spanò L. and Costantino P.(1985). Localisation of agropine-synthesing functions in the T- region of the root-inducing plasmid of *Agrobacterium rhizogenes* 1855. *Plasmid* **13**: 1-7

Diaz I., Moreno R. and Power J.B. (1988). Plant regeneration from protoplasts of *Capsicum annuum*. *Plant Cell Reports* **7**: 210-212

Dommisse E.M., Leung D.W.M., Shaw M.L. and Conner A.J. (1990). Onion as a monocotyledonous host for *Agrobacterium*. *Plant Science* **69**: 249-257

Ebida A.I-A. and Hu C-Y. (1993). *In vitro* morphogenetic responses and plant regeneration from pepper (*Capsicum annuum* L.cv.'Early California Wonder') seedling explants. *Plant Cell Reports* **13**: 107-110

Fári M. and Czakó M. (1981). Relationship between position and morphogenetic response of pepper hypocotyl explants cultured *in vitro*. *Scientia.Hortic.* **15**: 207-213

Fang G. and Grumet R. (1990). *Agrobacterium tumefaciens* -mediated transformation and regeneration of muskmelon plants. *Plant Cell Reports* **9**: 160-164

Fehér A., Felföldi K., Preiszner J. and Dudits D. (1991). PEG-mediated transformation of leaf protoplasts of *Solanum tuberosum* L. cultivars. *Plant Cell, Tissue and Organ Culture* **27**: 105-114

Filho E.S.F., Figueiredo L.F.A. and Monte-Neshich D.L. (1994). Transformation of potato (*Solanum tuberosum*) cv. 'Mantiqeira' using *Agrobacterium tumefaciens* and evaluation of herbicide resistance. *Plant Cell Reports* **13**: 666-670

Fraley R.T., Rogers S.G. and Horsch R.B. (1986). Genetic transformation in higher plants. In: CRC Critical Reviews in Plant Science, vol 4 ; Issue 1 (1-46)

Fraley R.T., Rogers S.G., Horsch R.B., Sanders P.R., Flick J.S. Adams S.P., Bittner M.L., Brand L.A., Fink C.L., Fry J.S., Gallupi G.R., Goldberg S.B., Hoffman N.L. and Woo S.C. (1983). Expression of bacterial genes in plant cells. *Proc. Natl. Acad. Sci. (USA)* **80**: 4803-4807

George L. and Narayanaswamy S. (1973). Haploid *Capsicum* through experimental androgenesis. *Protoplasma* **78**: 467-480

Gheysen G., van Montagu M. and Zambryski P. (1987). integration of *Agrobacterium tumefaciens* T-DNA involves rearrangements of target plant DNA sequences. *Proc. Natl. Acad. Sci. (USA)* **84**: 6169-6173

Gunay A.L. and Rao P.S. (1978). *In vitro* plant regeneration from hypocotyl and cotyledon explants of red pepper (*Capsicum*). *Plant Science Letters* **11**: 365- 372

Harini I. and Sita G.L. (1993). Direct somatic embryogenesis and plant regeneration from immature embryos of chilli (*Capsicum annuum* L.). *Plant Science* **89**: 107-112

Herrera-Estrella L., De Block M., Messens E., Hernalsteens J-P., van Montagu M. and Schell J. (1983). Chimeric genes as dominant selectable markers in plant cells. *Embo J.* **2**(6): 987-995

Hoekema A., Hirsch P.R., Hooykaas P.J.J. and Schilperoot R.A. (1983). A binary plant vector stratergy based on seperation of *vir* and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. *Nature* **303**: 179-180

Höfgen R. and Willmitzer L. (1988). Storage of competent cells for *Agrobacterium* transformation. *Nucleic Acid Research* **16** (20): 9877

Holden P.R. and Yeoman M.M. (1994). Variation in the growth and biosynthetic activity of cloned cell cultures of *Capsicum frutescens* and their response to an exogenously supplied elicitor. *Plant Cell Tissue and Organ Culture* **38**: 31-37

Holford P., Hernandez N. and Newbury H.J. (1992). Factors influencing the efficiency of T-DNA transfer during co-cultivation of *Antirrhinum majus* with *Agrobacterium tumefaciens*. *Plant Cell Reports* **11**: 196-199

Hooykaas P.J.J. (1989). Transformation of plant cells via *Agrobacterium*. *Plant Molecular Biology* **13**: 327-336

Horsch R.B., Fry J.E., Hoffman N.L., Eichholtz D., Rogers S.G. and Fraley R.T. (1985). A simple and general method for transferring genes into plants. *Science* **227**: 1229-1231

Huffman G.A., White F.F., Gordan M.P. and Nester E.W. (1984). Hairy root inducing plasmid:physical map and homology to tumour inducing plasmids. *Journal of Bacteriology* **157**: 269-270

James D.J., Passey A.J. and Barbara D.J. (1990). *Agrobacterium* mediated transformation of the cultivated strawberry *FagariaxAnanmassa* Dutch. using disarmed binary vectors. *Plant Science* **69**: 79-94

Jefferson R.A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Molecular Biology Reporter* **5**(4): 387-405

Jefferson R.A., Kavanagh T.A. and Bevan M.W. (1987). GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.***6** (13): 3901-3907

Johnson-Sudhaker T., Ravishanker G.A. and Venkataraman L.V. (1990). *In vitro* capsaicin production by immobilized cells and placental tissues of *Capsicum annuum* L. grown in liquid medium. *Plant Science* **70**: 223-229

Klee H., Horsch R.B. and Rogers S.B. (1987). *Agrobacterium*-mediated plant transformation and its further applications to plant biology. *Annual Review of Plant Physiology* **38**: 467-486

Kosugi S., Ohashi Y., Nakajima K. and Arai Y. (1990). An improved assay for β–glucuronidase in transformed cells: methanol almost completely supresses a putative endogenous β-glucuronidase activity. *Plant Science* **70**: 133-140

Lichtenstein C. (1986). A bizzare vegetable bestiality. *Nature* **322**: 682-683

Lichtenstein C.P. and Fuller S.L. (1987). Vectors for the genetic engineering of plants. *Genetic Engineering* **6**: 103-183

Liu W., Parrott W.A., Hilderbrand D.F., Collins G.B. and Williams E.G. (1990). *Agrobacterium* induced gall formation in bell pepper (*Capsicum annuum* L.) and formation of shoot-like structures expressing introduced genes. *Plant Cell Reports* **9**: 360-364

Maniatis T., Fritsch E.F. and Sambrook J. (1982). *Molecular cloning, a laboratory manual.* Cold Spring Harbour Press, Cold Spring Harbour, New York. 545p.

McCormick S., Niedermeyer J.F., Barnason, A., Horsch R. and Fraley R. (1986). Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens. Plant Cell Reports* **5**: 81-84

Miljuš-Djukié J., Nešković M., Ninković G. and Crkvenjakov R. (1992). *Agrobacterium*-mediated transformation and plant regeneration of buckwheat (*Fagopyrum esculentum* Moench) . *Plant cell Tissue and Organ Culture* **29**:101-108

Morrison R.A., Koning R.E. and Evans D.A. (1986). Pepper.In: *Handbook of Plant Cell Culture*.vol 4. (Evans D.A., Sharp W.R. and Ammirato P.V. eds.) p 552-573

Murashige T. (1974). Plant propagation through tissue cultures. *Annual Review Plant Physiology* **25**: 135-166

Murashige T. and Skoog F. (1962). A revised medium for rapid growth and bioassays of tobacco tissue cultures. *Physiologia Plantarum* **15**: 473-497

Murphy J.F. and Kyle M.M.(1994). Isolation and viral infection of *Capsicum* leaf protoplasts. *Plant Cell Reports* **13**: 397-400

Neff N.T. and Binns A.N. (1985). *Agrobacterium tumefaciens* interaction with suspension-cultured tomato cells. *Plant Physiology* **77**: 35-42

Ohta S., Mita S., Hattori T. and Nakamura K. (1990). Construction and expression in tobacco of a β-glucuronidase (GUS) reporter gene containing an intron within the coding sequence. *Plant Cell Physiology* **31**(6): 805-813

Özcan S., Firek S. and Draper J. (1993). Selectable marker genes engineered for specific expression in target cells for plant transformation. *Bio/Technology* **11**: 218-221

Pang S.Z. and Sanford J.C. (1988). *Agrobacterium*-mediated gene transfer in papaya. *Journal of the American Society of Horticultural Science* **113**: 287-291

Phillips G.C. and Hubstenberger J.F. (1985). Organogenesis in pepper tissue cultures. *Plant Cell Tissue and Organ Culture* **4**: 261-269

Pollock K., Barfield D.J. and Shields R. (1983). The toxicity of antibiotics to plant cell cultures. *Plant Cell Reports* **2**: 36-39

Potrykus I. (1991). Gene transfer to plants: Assesment of published approaches and results. *Annual Review of Plant Physiology and Plant Molecular Biology* **42**: 205-225

Potrykus I. (1990). Gene transfer to plants : assesment and perspectives. *Physiologia Plantarum* **79**: 125-134

Radke S.E., Andrews B.M., Moloney M.M., Crouch M.L., Kridl J.C. and Knauf V.C. (1988). Transformation of *Brassica napus* L. using *Agrobacterium tumefaciens*: developmentally regulated expression of a reintroduced napin gene. *Theor. Appl. Genet.* **75**: 685-694

Rakousky s. and Matousek J. (1994). Direct organogenesis in hop: a pre-requisite for an application of *A. tumefaciens*- mediated transformation. *Biologia Plantarum.* **36** (20): 191-200

Ramage C. *An investigation of shoot formation in* Capsicum annuum *L. var. 'Sweet banana'*. Christchurch, University of Canterbury, 1994. 137p.

Reynolds J.F. (1986). Regeneration in vegetable species. In: *Cell Culture and Somatic Cell Genetics of Plants.* Volume 3, 151-178.Ed; Vasil, I.K. Acad.Press.

Rotino G.L. and Gleddie S. (1990). Transformation of eggplant (*Solanum melongena* L.) using a binary *Agrobacterium tumefaciens vector. Plant Cell Reports* **9**: 26-29

Sahi S.V., Gagliardo R.W., Chilton M-D. and Chilton W.S. (1994). A thin layer chromotographic technique for detecting inducers of *Agrobacterium* virulence genes in corn, wheat and rye. *Plant Cell Reports* **13**: 489-492

Sangwan R.S., Bourgeois Y. and Sangwan-Noreel B. (1991). Genetic transformation of *Arabidopsis thaliana* zygotic embryos and identification of critical parameters influencing tranformation efficiency. *Molecular General Genetics* **230**: 475-485

Saxena P.K., Gill R., Rashid A. and Maheshwari S.C.(1981). Isolation and culture of protoplasts of *Capsicum annuum* L. and their regeneration into plants flowering *in vitro*. *Protoplasma* **108**: 357-360

Schmidt R. and Willmitzer L. (1988). High efficiency *Agrobacterium tumefaciens* mediated transformation of *Arabidopsis thaliana* leaf and cotyledon explants. *Plant Cell Reports* 7: 583-586

Skoog F. and Miller C.O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symp. Soc. Exp.Biol.*, **11**: 118-131

Slusarenko A.J. (1990). A rapid mini-prep for the isolation of total DNA from *Agrobacterium tumefaciens* . *Plant Molecular Biology Reporter* 8(4): 249-252

Sripichitt P., Nawata E. and Shigenaga S. (1987). *In vitro* shoot-forming capacity of cotyledon explants in red pepper (*Capsicum annuum* L.cv.'Yatsufusa'). *Japan Journal of Breeding* **37**: 133-142

Stachel S.E., Nester E.W. and Zambryski P. (1986a). A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression. *Proc. Natl. Acad. Sci.(USA)* **83**: 379-383

Stachel S.E. and Zambryski P.G. (1986b). *Agrobacterium tumefaciens* and the susceptible plant cell: a novel adaption of extracellular recognition and DNA conjugation. *Cell* **47**: 155-157

Stachel S.E., Messens E., van Montagu M. and Zambryski P. (1985). Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. *Nature* **318**: 624-629

Tepfer D. (1990). Genetic transformation using *Agrobacterium rhizogenes* . *Physiologia Plantarum* **79**: 140-146

Tepfer G. (1984). Transformation of several species of higher plants by *Agrobacterium rhizogenes* : Sexual

transmission of the transformed genotype and phenotype. *Cell* **37**: 959-967

Thorpe T.A. and Patel K.R. (1984). Clonal propagation: Adventitious bud. In Cell Culture and Somatic Genetics of Plants. Ed.,Vasil, I.K. Acad.Press. pp49-60

Valero-Montero L.L. and Ochoa-Alejo N. (1992). A novel approach for chili pepper (*Capsicum annuum* L.) plant regeneration: shoot induction in rooted hypocotyls. *Plant Science* **84** (2)**:** 215-219

Vancanneyt G., Schmidt R., O'Conner-Sanchez A., Willmitzer L. and Rocha-Sosa M. (1990). Construction of an intron-containing marker gene: splicing the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. *Molecular General Genetics* **220**: 245-250

van Wordragen M.F. and Dons H.J.M. (1992). *Agrobacterium tumefaciens* -mediated transformation of recalcitrant crops. *Plant Molecular Biology Reporter* **10**(1): 12-36

Vasil V., Castillo A.M., Fromm M.E. and Vasil I.K. (1992). Herbicide resitant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryonic callus. *Bio/Technology* **10**: 667-674

Voisey C.R., White D.W.R., Dudas B., Appleby R.D., Ealing P.M. and Scott A.G. (1994). *Agrobacterium*-mediated transformation of white clover using direct shoot organogenesis. *Plant Cell Reports* **13**: 309-314

Wang K., Genetello C., van Montagu M. and Zambryski P. (1987). Sequence context of the T-DNA border repeat element determines its relative activity during T-DNA transfer to plant cells. *Molecular General Genetics* **210**: 338-346

Wang K., Herrera-Esterella L., van Montgu M. and Zambryski P. (1984). Right 25bp terminus sequence of the nopaline T-DNA is essential for and determines direction of DNA transfer from *Agrobacterium* to the plant genome. *Cell* **38**: 445-462

Wilmink A. and Dons J.J.M. (1993). Selective agents and marker genes for use in transformation of monocotyledonous plants. *Plant Molecular Biology Reporter* **11**(2):165-185

Willmitzer L., De Beuckeleer M., Lremmers M., van Montagu M. and Schell G. (1980). DNA from Ti plasmid present in nucleus and absent from plastids of crown gall plant cells. *Nature* **287**: 359-361

Winans S.C. (1987). Characterization of the *vir*E operon of the *Agrobacterium tumefaciens* plasmid pTiA6 *Nucleic Acid Research* **15**: 825-837

Yenofsky M.F. (1986). The *vir*D operon of *Agrobacterium tumefaciens* encodes a site specific endonuclease. *Cell* **47**: 471-477

Yepes L.M. and Aldwinkle H.S. (1994). Factors that affect leaf regeneration efficiency in apple and effect of antibiotics in morphogenesis. *Plant cell tissue and organ culture* **37** (3): 257-269

Zambryski P.C. (1992). Chronicles from the *Agrobacterium*-plant cell DNA transfer story. *Annual Review of Plant Physiology and Plant Molecular Biology* **43**: 465-490

Zambryski P.C., Joos H., Genetello C., Leemans J., van Montagu M. and Schell J. (1983). Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. *EMBO J.*2(12); 2143-2150

ACKNOWLEDGEMENT

I wish to thank my supervisor, Dr. David Leung for his patience, advice and help during my research. I also wish to thank Karen Baas, Barbara Lloyd, fellow students of the Plant Biotechnology laboratory, Dougal Holmes for photography and Dave Condour for nurturing my plants in the glasshouses.

Special thanks to Jace Carson for his careful and critical reading of my thesis and for sharing with me his expertise with computers.

I wish to express my sincere gratitude to my family for their support and encouragment and to my husband Ravindranath, I remain forever grateful.

APPENDIX A

MURASHIGE AND SKOOG (MS) STOCK SOLUTIONS

Major Salts (10X)	**gL^{-1}**
NH_4NO_3	16.5
KNO_3	19.0
$CaCl_2.2H_2O$	4.4
$MgSO_4.7H_2O$	3.7
KH_2PO_4	1.7

Stored at 4°C

Minor Salts (100X)	**gL^{-1}**
KI	0.166
H_3BO_3	1.24
$MnSO_4.7H_2O$	4.46
$ZnSO_4.7H_2O$	1.72
$CuSO_4.5H_2O$	0.005
$CoCl_2.6H_2O$	0.005
$Na_2MoO_4.2H_2O$	0.05

Stored at 4°C

Organic supplement (100X)	**mgL^{-1}**
myo-inisitol	10 000
nicotinic acid	50
pyridoxine-HCl	50
thiamine-HCl	50
glycine	200

Stored at 4°C

Iron stock (100X)

Solution A:	$FeSO_4.7H_2O$	1.39g in 200ml dH_2O
Solution B:	$Na_2EDTA.2H_2O$	1.865g in 200ml dH_2O

Solutions A and B were mixed, adjusted to pH 5.8 and made upto a volume of 500ml and stored at 4°C in a dark bottle.

1 litre of MS medium contains 100ml of major salts, 10ml of minor salts, 10ml organic supplement and 10ml iron stock.

APPENDIX B

PREPARATION OF GROWTH REGULATORS

Benzylaminopurine (BA) stock solution

0.1mgml^{-1} in distilled water, dissolved initially in 1N NaOH and stored at 4°C.

Indole-butyric-acid (IBA) stock solution

0.1mgml^{-1} in distilled water, dissolved initially in 1N NaOH and stored at 4°C.

Napthalene acetic acid (NAA) stock solution

0.1mgml^{-1} in distilled water, dissolved initially in 1N NaOH and stored at 4°C.

APPENDIX C

ANTIBIOTICS

Claforan

Stock solution: 200mg ml^{-1} in distilled water , filter sterilized using millipore filter with diameter 0.22 μm and stored in aliquots at 20°C.

Kanamycin

Stock solution: 25mg ml^{-1} in distilled water , filter sterilized using millipore filter with diameter 0.22 μm and stored in aliquots at 20°C.

APPENDIX D

CULTURE MEDIUM FOR *AGROBACTERIUM* AND *E.COLI*

***Luria-Bertani* (LB) medium**	**gL^{-1}**
Bactotryptone	10
Yeast extract	5
NaCl	10
Agar (for solid medium only)	15

LB medium was used at pH 7.0

APPENDIX E

GUS ASSAY:

Histochemical staining with the substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-GLUC)

5mg X-GLUC was dissolved in 50µl dimethyl formadide. To this 20% (v/v) distilled methanol was added. This solution was made to 10ml with the addition of 50mM $NaPO_4$ (pH 7).

Fresh tissue was incubated in this solution overnight.

APPENDIX F

Results from studies carried out to establish a regeneration protocol of shoots induced from the hypocotyl explant of *Capsicum annuum* L. ('Sweet banana'). The growth medium used was MS containing 3%(w/v) sucrose and a combination of plant growth regulators IBA and NAA. The production of shoot, root or callus on each explant was observed after 4 weeks and denoted as :
{+++}- significantly predominant; {++}- moderate; {+}- significantly less; {-}- absent.

Control

Treatment (mgL^{-1})		Replicate number	Response		
IBA	NAA		shoot	root	callus
0	0	1	++	-	++
		2	++	-	-
		3	+	-	-
		4	+	-	-
		5	++	-	+
		6	+	-	-
		7	++	-	-
		8	++	-	-
		9	++	-	-
		10	+	-	-

Treatment (mgL^{-1})		Replicate number	Response		
IBA	NAA		Shoot	root	callus
0.05	0.1	1	+	++	+++
		2	+	+	+++
		3	+	-	++
		4	+	-	++
		5	+	-	++
		6	+	-	++
		7	+	-	++
		8	+	+	+++
		9	+	-	++
		10	-	-	+++
		11	++	++	+++
		12	++	++	+++

Treatment (mgL^{-1})		Replicate number	Response		
IBA	NAA		shoot	root	callus
0	0.1	1	+	+	++
		2	+	-	++
		3	+	-	++
		4	+	-	++
		5	*		
		6	-	-	+
		7	-	-	+
		8	+	-	++
		9	+	-	+
		10	-	-	++

* did not survive

Treatment (mgL^{-1})		Replicate number	Response		
IBA	NAA		shoot	root	callus
0.02	0.05	1	+++	+	-
		2	++	+	-
		3	+++	+	-
		4	*		
		5	+++	-	-

Treatment (mgL^{-1})		Replicate number	Response		
IBA	NAA		shoot	root	callus
0.01	0.1	1	++	-	-
		2	++	-	-
		3	++	-	-
		4	+++	-	-
		5	++	-	-

Treatment (mgL^{-1})		Replicate number	Response		
IBA	NAA		shoot	root	callus
0.02	0.1	1	+++	-	-
		2	+++	-	-
		3	+++	-	-
		4	+++	-	-
		5	+++	-	-

* did not survive

Treatment (mgL^{-1})		Replicate number	Response		
IBA	NAA		shoot	root	callus
0.01	0.2	1	++	-	+
		2	++	-	-
		3	++	-	+
		4	++	-	+
		5	++	-	+

Treatment (mgL^{-1})		Replicate number	Response		
IBA	NAA		shoot	root	callus
0.02	0.2	1	++	-	-
		2	++	-	-
		3	++	-	-
		4	++	-	-
		5	++	-	-

Treatment (mgL^{-1})		Replicate number	Response		
IBA	NAA		shoot	root	callus
0.1	0.05	1	*		
		2	*		
		3	++	-	-
		4	++	-	-
		5	+	+	-
		6	++	-	-
		7	*		

* did not survive

Treatment (mgL^{-1})		Replicate number	Response		
IBA	NAA		shoot	root	callus
0.02	0	1	+	-	+
		2	+	-	+
		3	+	-	+
		4	+	-	+
		5	+	-	+

Treatment (mgL^{-1})		Replicate number	Response		
IBA	NAA		shoot	root	callus
0.01	0	1	+	-	+
		2	+	-	+
		3	+	-	+
		4	+	-	++
		5	+	-	-

Treatment (mgL^{-1})		Replicate number	Response		
IBA	NAA		shoot	root	callus
0.5	0.0	1	++	-	-
		2	*		
		3	++	-	++
		4	++	-	++
		5	*		

* did not survive

Treatment (mgL^{-1})		Replicate number	Response		
IBA	NAA		shoot	root	callus
0.5	0.05	1	*		
		2	++	+	-
		3	*		
		4	+	+	-
		5	++	+	-

Treatment (mgL^{-1})		Replicate number	Response		
IBA	NAA		shoot	root	callus
0.05	0.05	1	+	-	+
		2	+	-	++
		3	+	-	++
		4	+	-	+
		5	+	-	+

Treatment (mgL^{-1})		Replicate number	Response		
IBA	NAA		shoot	root	callus
0.01	0.05	1	-	++	++
		2	-	-	++
		3	+	+	+++
		4	+	-	++
		5	-	-	++

* did not survive

Treatment (mgL^{-1})		Replicate number	Response		
IBA	NAA		shoot	root	callus
0	0.05	1	-	++	+
		2	+	-	+
		3	+	-	+
		4	*		
		5	+	-	+

Treatment (mgL^{-1})		Replicate number	Response		
IBA	NAA		shoot	root	callus
0.01	0	1	++	-	+
		2	++	-	+
		3	++	-	+
		4	++	-	+
		5	++	-	+

* did not survive

APPENDIX G

Experiment number: 1

Variety of *Capsicum annuum* L. : 'Sweet banana'

Explant: upper hypocotyl

Strain of *Agrobacterium* : overnight culture of C58::pBI 121

Control: SIM

Co-cultivation: explants were inoculated with *Agrobacterium* for 60 minutes at 26(±1)°C

Selection media: SIM+K+C for 20 days at 26(±1)°C in light

Results:

	Response	Replicates
control	no shoot formation	10
C58::pBI 121	no shoot formation	30

Experiment number: 2 (repeat of experiment number 1)

Variety of *Capsicum annuum* L. : 'Sweet banana'

Explant: upper hypocotyl

Strain of *Agrobacterium* : overnight culture of C58::pBI 121

Control: SIM

Co-cultivation: explants were inoculated with *Agrobacterium* for 60 minutes at 26(±1)°C

Selection media: SIM+K+C for 20 days at 26(±1)°C in light

Results:

	Response	Replicates
control	no shoot formation	10
C58::pBI 121	no shoot formation	30

Experiment number: 3

Variety of *Capsicum annuum* L. : 'Sweet banana'

Explant: upper hypocotyl

Strain of *Agrobacterium* : LBA4404::pBI 121

Control: SIM

Co-cultivation: explants were inoculated with *Agrobacterium* for 30 minutes at 26(±1)°C

Selection media: SIM+K+C for 20 days at 26(±1)°C in light
(10 control replicates were transferred to SIM without antibiotics)

Results:

	Response	Replicates
control (SIM)	6 formed shoots	10
control (SIM+K+C)	0	10
LBA4404::pBI 121	0	30

Experiment number: 4

Variety of *Capsicum annuum* L. : 'Sweet banana'

Explant: upper hypocotyl

Strain of *Agrobacterium* : overnight culture of C58::pBI 121

Control: SIM

Co-cultivation: explants were inoculated with *Agrobacterium* for 30 minutes at 26(±1)°C and transferred to IM or IM+K+C for 72 hours at 26(±1)°C in light

Selection media: SIM+K+C for 20 days at 26(±1)°C in light

Results:

	Number of upper hypocotyl explants forming shoots	
	IM	IM+K+C
control	1 (10)[1]	2 (10)
C58::pBI 121 (1[a])	1 (10)	2 (10)
C58::pBI 121 (0.2[b])	0 (10)	3 (10)
C58::pBI 121 (0.5[c])	4 (10)	3 (10)

Histochemical GUS activity in shoots formed:

	GUS staining
Control	negative
all C58::pBI 121 experiments	negative

[1] number of replicates
[a] Overnight culture of *Agrobacterium* pelleted and resuspended in 10ml SIM
[b] 0.2 dilution of overnight culture that was pelleted and resuspended in 10ml SIM
[c] 0.5 dilution of overnight culture that was pelleted and resuspended in 10ml SIM

Experiment number:	5
Variety of *Capsicum annuum* L.	'Sweet banana'
Explant:	upper hypocotyl
Strain of *Agrobacterium* :	overnight culture of A4T::pBI 121
Control:	SIM
Co-cultivation:	explants were inoculated with *Agrobacterium* for 30 minutes at 26(±1)°C and transferred to IM or IM+K+C for 72 hours at 26(±1)°C in light
Selection media:	SIM+K+C for 20 days at 26(±1)°C in light

Results:

	Number of upper hypocotyl explants forming shoots	
	IM	IM+K+C
control	0 (10)[1]	0 (10)
A4T::pBI 121 (1[a])	0 (10)	0 (10)
A4T::pBI 121 (0.2[b])	0 (10)	0 (10)
A4T::pBI 121 (0.5[c])	0 (10)	0 (10)

[1] number of replicates
[a] Overnight culture of *Agrobacterium* pelleted and resuspended in 10ml SIM
[b] 0.2 dilution of overnight culture that was pelleted and resuspended in 10ml SIM
[c] 0.5 dilution of overnight culture that was pelleted and resuspended in 10ml SIM

Experiment number: 6

Variety of *Capsicum annuum* L. : 'Sweet banana'

Explant: upper hypocotyl

Strain of *Agrobacterium* : overnight culture of C58::pBI 121

Control: SIM

Co-cultivation: explants were inoculated with *Agrobacterium* for 30 minutes at 26(±1)°C and transferred to IM+K+C for 48 hours at 26(±1)°C in light

Selection media: SIM+K+C for 20 days at 26(±1)°C in light

Results:

	Number of hypocotyls forming shoots	
	dark	light
control		0 (10)[1]
C58::pBI 121		0 (60)

This experiment was repeated with the same number of explants and the results were negative.

[1] number of replicates

Experiment number: 7

Variety of *Capsicum annuum* L. : 'Sweet banana'

Explant: upper hypocotyl

Strain of *Agrobacterium* : overnight culture of LBA4404::pBI 121

Control: SIM

Co-cultivation: explants were inoculated with *Agrobacterium* for 30 minutes at 26(±1)°C and transferred to IM for 72 hours

Selection media: SIM+K+C for 20 days at 26(±1)°C in light

Results:

	number of hypocotyls forming shoots
control	0 (10)[1]
LBA4404::pBI 121	2 (30)

Histochemical GUS activity in shoots formed:

	GUS staining
LBA4404::pBI 121	negative

[1] number of replicates

Experiment number: 8

Variety of *Capsicum annuum* L. : 'Sweet banana'

Explant: upper hypocotyl

Strain of *Agrobacterium* : overnight culture of C58::pBI 121

Control: SIM

Co-cultivation: explants were inoculated with *Agrobacterium* for 30 minutes at 26(±1)°C and transferred to IM or IM+K+C for 72 hours at 26(±1)°C in light

Selection media: SIM+K+C for 20 days at 26(±1)°C in light

Results:

	Number of upper hypocotyl explants forming shoots	
	IM	IM+K+C
control	0 (10)[1]	0 (10)
C58::pBI 121 (1[a])	0 (20)	0 (20)
C58::pBI 121 (0.2[b])	1 (20)	3 (20)
C58::pBI 121 (0.5[c])	1 (20)	2 (20)

Histochemical GUS activity in shoots formed:

	GUS staining
all C58::pBI 121 experiments	negative

[1] number of replicates
[a] Overnight culture of *Agrobacterium* pelleted and resuspended in 10ml SIM
[b] 0.2 dilution of overnight culture that was pelleted and resuspended in 10ml SIM
[c] 0.5 dilution of overnight culture that was pelleted and resuspended in 10ml SIM

Experiment number: 9

Variety of *Capsicum annuum* L. : 'Sweet banana'

Explant: upper hypocotyl

Strain of *Agrobacterium* : overnight culture of C58::pBI 121

Control: SIM

Co-cultivation: explants were inoculated with *Agrobacterium* for 30 minutes at 26(±1)°C and transferred to IM+C for 72 hours at 26(±1)°C in light

Selection media: SIM+K+C for 20 days at 26(±1)°C in light

Results:

	Number of upper hypocotyl explants forming shoots IM
control	1 (10)[1]
C58::pBI 121	17 (60)

Histochemical GUS activity in shoots formed:

	GUS staining
control	negative
C58::pBI 121	negative

[1] number of replicates

Experiment number: 10

Variety of *Capsicum annuum* L. : 'Sweet banana'

Explant: upper hypocotyl

Strain of *Agrobacterium* : overnight culture of C58::pBI 121

Control: SIM

Co-cultivation: explants were inoculated and kept in the presence of the *Agrobacterium* for 30 minutes at 26(±1)°C

Selection media: SIM+K+C for 20 days at 26(±1)°C in light

Results :

	Number of upper hypocotyl explants forming shoots
control	0 (10)[1]
C58::pBI 121	0 (40)

[1] number of replicates

In the following experiments the *Agrobacterium* strains contained the plasmid pIG 121. After co-cultivation the explants were tested for GUS activity without the selection for antibiotic resistance..

Experiment number:	11
Variety of *Capsicum annuum* L. :	'Sweet banana'
Explant:	upper hypocotyl pre-conditioned by culturing in SIM at 26(±1)°C in light for 4 days
Strain of *Agrobacterium* :	overnight culture of A4T::pIG 121 , C58::pIG 121 and LBA4404::pIG 121
Control:	SIM
Co-cultivation:	explants were inoculated with *Agrobacterium* for 60 minutes at 26(±1)°C in the dark and transferred to IM+C for 24 hours at 26(±1)°C in light

Results:

	GUS staining
control	negative (10)[1]
A4T::pIG 121	negative (20)
C58::pIG 121	negative (20)
LBA4404::pIG 121	negative (20)

[1] number of replicates

Experiment number:	12
Variety of *Capsicum annuum* L. :	'Sweet banana'
Explant:	upper hypocotyl pre-conditioned by culturing in SIM at 26(±1)°C in light for 4 days
Strain of *Agrobacterium* :	overnight culture of A4T::pIG 121 , C58::pIG 121 and LBA4404::pIG 121
Control:	SIM
Co-cultivation:	explants were inoculated with *Agrobacterium* for 60 minutes at 26(±1)°C and transferred to IM+C for 48 hours at 26(±1)°C in light

Results:

	GUS staining
control	negative (10)[1]
A4T::pIG 121	negative (20)
C58::pIG 121	negative (20)
LBA4404::pIG 121	negative (20)

[1] number of replicates

Experiment number: 13

Variety of *Capsicum annuum* L. : 'Sweet banana'

Explant: upper hypocotyl pre-conditioned by culturing in SIM at 26(±1)°C in light for 4 days

Strain of *Agrobacterium* : overnight culture of A4T::pIG 121 , C58::pIG 121 and LBA4404::pIG 121

Control: SIM

Co-cultivation: explants were inoculated with *Agrobacterium* for 60 minutes at 26(±1)°C and transferred to IM+C for 72 hours at 26(±1)°C in light

Results:

	GUS staining
control	negative (10)[1]
A4T::pIG 121	negative (20)
C58::pIG 121	negative (20)
LBA4404::pIG 121	negative (20)

[1] number of replicates

Experiment number: 14

Variety of *Capsicum annuum* L. : 'Sweet banana'

Explant: cotyledons, roots, upper and lower hypocotyl of 11-day old seedling

Strain of *Agrobacterium* : overnight culture of A4T::pIG 121 , C58::pIG 121 and LBA4404::pIG 121

Control: SIM

Co-cultivation: explants were inoculated with *Agrobacterium* for 60 minutes at 26(±1)°C and transferred to IM+C for 48 hours at 26(±1)°C in light

Results:

	GUS staining			
10 replicates for each trial	upper hypocotyl	lower hypocotyl	cotyledon	root
control	negative	negative	negative	negative
A4T::pIG 121	negative	negative	negative	negative
C58::pIG 121	negative	negative	negative	negative
LBA4404::pIG 121	negative	negative	negative	negative

Experiment number: 15

Variety of *Capsicum annuum* L. : 'Sweet banana'

Explant: leaf segments, stem sections, petals and anthers of mature plant

Strain of *Agrobacterium* : overnight culture of C58::pIG 121 and LBA4404::pIG 121

Control: SIM

Co-cultivation: explants were inoculated with *Agrobacterium* for 60 minutes at 26(±1)°C and transferred to IM+C for 48 hours at 26(±1)°C in light

Results:

	GUS staining			
	leaf	stem	petals	anthers
control	negative(2)[a]	negative(2)	negative(2)	negative(2)
C58::pIG 121	positive(2/10)[b]	positive (7/10)	positive(1/5)	positive(2/5)
LBA4404::pIG 121	positive(2/10)	positive(3/10)	negative	negative

[a] number of replicates
[b] number of explants with positive GUS response per total number of explants assayed

Experiment number: 16

Variety of *Capsicum annuum* L. : *Capsicum annuum* L. ('Yolo Wonder')

Explant: petals and anthers from mature plant

Strain of *Agrobacterium* : overnight culture of C58::pIG 121 and LBA4404::pIG 121

Control: SIM

Co-cultivation: explants were inoculated with *Agrobacterium* for 60 minutes at 26(±1)°C and transferred to IM+C for 48 hours at 26(±1)°C in light

Results:

	GUS staining	
	petal	anther
control	negative(2)	negative(2)
C58::pIG 121	positive	positive
LBA4404::pIG 121	positive	positive

Experiment number: 17

Variety of *Capsicum annuum* L. : 'Sweet banana'

Explant: leaf segments and stem sections from plants that were 2,4,8 and 10 weeks old

Strain of *Agrobacterium* : overnight culture of A4T::pBI 121 , C58::pIG 121 and LBA4404::pIG 121

Control: SIM

Co-cultivation: explants were inoculated with *Agrobacterium* for 60 minutes at 26(±1)°C and transferred to IM+C for 48 hours at 26(±1)°C in light

Results:

	GUS staining			
Age of plant (weeks)	2	4	8	10
A4T::pIG 121	negative	negative	negative	negative
C58::pIG 121	negative	negative	negative	negative
LBA4404::pIG 121	negative	negative	negative	negative

Experiment number: 18

Variety of *Capsicum annuum* L. : 'Sweet banana'

Explant: upper hypocotyl pre-conditioned by culturing in SIM for 8 days

Strain of *Agrobacterium* : overnight culture of A4T::pIG 121 , C58::pIG 121 and LBA4404::pIG 121

Control: SIM

Co-cultivation: explants were inoculated with *Agrobacterium* for 60 minutes at 26(±1)°C and transferred to IM+C for 48 hours at 26(±1)°C in light

Results:

	GUS staining
control	negative (10) [1]
A4T::pIG 121	negative (20)
C58::pIG 121	negative (20)
LBA4404::pIG 121	negative (20)

[1] number of replicates

Experiment number: 19

Variety of *Capsicum annuum* L. : 'Sweet banana'

Explant: upper hypocotyl pre-conditioned by culturing in SIM+K for 4 days

Strain of *Agrobacterium* : overnight culture of A4T::pIG 121 , C58::pIG 121 and LBA4404::pIG 121

Control: SIM

Co-cultivation: explants were inoculated with *Agrobacterium* for 60 minutes at 26(±1)°C and transferred to IM+C for 48 hours at 26(±1)°C in light

Results:

	GUS staining
control	negative (10) [1]
A4T::pIG 121	negative (20)
C58::pIG 121	negative (20)
LBA4404::pIG 121	negative (20)

[1] number of replicates

Experiment number: 20

Variety of *Capsicum annuum* L. : 'Sweet banana'

Explant: upper hypocotyl

Strain of *Agrobacterium* : overnight culture of A4T::pIG 121 , C58::pIG 121 and LBA4404::pIG 121

Control: SIM

Co-cultivation: explants were inoculated with *Agrobacterium* for 60 minutes at 26(±1)°C and transferred to IM+C for 48 hours at 26(±1)°C in light

Results:

	GUS staining
control	negative (10) [1]
A4T::pIG 121	negative (20)
C58::pIG 121	negative (20)
LBA4404::pIG 121	negative (20)

[1] number of replicates

Experiment number: 21

Variety of *Capsicum annuum* L. : 'Sweet banana'

Explant: leaf segments, stem sections, petals and anthers of mature plant

Strain of *Agrobacterium* : overnight culture of A4T::pIG 121 , C58::pIG 121 and LBA4404::pIG 121

Control: SIM

Co-cultivation: explants were inoculated with *Agrobacterium* for 60 minutes at 26(±1)°C and transferred to IM+C for 48 hours at 26(±1)°C in light

Results:

	GUS staining			
	leaf	stem	petals	anthers
control	negative	negative	negative	negative
A4T::pIG 121	negative	negative	negative	negative
C58::pIG 121	positive	positive	positive	positive
LBA4404::pIG 121	positive	positive	positive	positive

(Percentages of explants transformed or recorded.)

Experiment number: 22

Variety of *Capsicum annuum* L. : 'Sweet banana'

Explant: petals and anthers of mature plant

Strain of *Agrobacterium* : overnight culture of A4T::pIG 121 , C58::pIG 121 and LBA4404::pIG 121

Control: SIM

Co-cultivation: explants were inoculated with *Agrobacterium* for 60 minutes at 26(±1)°C and transferred to IM+C for 48 hours at 26(±1)°C in light

Results:

	GUS staining	
	petals	anthers
control	negative	negative
A4T::pIG 121	negative	negative
C58::pIG 121	positive (3/10)[#]	positive(3/10)
LBA4404::pIG 121	negative	negative

[#] number of explants with positive GUS staining per total number of replicates

Experiment number: 23

Variety of *Capsicum annuum* L. : 'Sweet banana'

Explant: petals and anthers of flowers at 3 different stages from mature plant

Strain of *Agrobacterium* : overnight culture of A4T::pIG 121 , C58::pIG 121 and LBA4404::pIG 121

Control: SIM

Co-cultivation: explants were inoculated with *Agrobacterium* for 60 minutes at 26(±1)°C and transferred to IM+C for 48 hours at 26(±1)°C in light

Results:

	GUS staining		
	unopened	partly opened	fully opened
A4T::pIG 121	negative	negative	negative
C58::pIG 121	negative	negative	negative
LBA4404::pIG 121	negative	negative	negative